Differential impact of acute and prolonged cAMP agonist exposure on protein kinase A activation and human myometrium contractile activity.

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KEY POINTS SUMMARY

- Over 15 million babies are born prematurely each year with approximately 1 million of these babies dying as a direct result of preterm delivery.
- β_2-adrenoreceptor agonists that act via cAMP can reduce uterine contractions to delay preterm labour, but their ability to repress uterine contractions lasts ≤ 48 hours and their use does not improve neonatal outcomes.
- Previous research has suggested that cAMP inhibits myometrial contractions via protein kinase A (PKA) activation, but this has yet to be demonstrated with PKA-specific agonists.
- We investigated the role of PKA in mediating cAMP-induced human myometrial relaxation, and the impact of prolonged cAMP elevation on myometrial contractility.
- Our findings suggest that PKA is not the sole mediator of cAMP-induced myometrial relaxation and that prolonged prophylactic elevation of cAMP alone is unlikely to prevent preterm labour (PTL).

ABSTRACT

Acute cAMP elevation inhibits myometrial contractility, but the mechanisms responsible are not fully defined and the long-term effects uncertain. These need to be defined in pregnant human myometrium before the therapeutic potential of cAMP-elevating agents in the prevention of preterm labour can be realised. In the present study, we tested the hypotheses that PKA activity is necessary for cAMP-induced myometrial relaxation and prolonged cAMP elevation can prevent myometrial contractions. Myometrial tissues obtained from term, pre-labour elective Caesarean sections were exposed to receptor-independent cAMP agonists to determine the relationship between myometrial contractility (spontaneous and oxytocin-induced), PKA activity, HSP20 phosphorylation and expression of contraction-associated and cAMP signalling proteins. Acute (1 h) application of cAMP agonists

promoted myometrial relaxation but this was weakly related to PKA activation. PKA-specific activator, 6-Bnz-cAMP, increased PKA activity (6.8 ± 2.0 mean fold *versus* vehicle; p = 0.0313) without inducing myometrial relaxation. Spontaneous myometrial contractility declined after 24 hours but was less marked when tissues were constantly exposed to cAMP agonists, especially for 8-bromo-cAMP (4.3 ± 1.2 mean fold *versus* vehicle; p = 0.0043); this was associated with changes to calponin, cofilin and HSP20 phosphorylated/total protein levels. Oxytocin-induced contractions were unaffected by pre-incubation with cAMP agonists despite treatments being able to enhance PKA activity and HSP20 phosphorylation. These data suggest that cAMP-induced myometrial relaxation is not solely dependent on PKA activity and the ability of cAMP agonists to repress myometrial contractility is lost with prolonged exposure. We conclude that cAMP agonist treatment alone may not prevent preterm labour.

ABBREVIATIONS. 6-Bnz-cAMP, N6-Benzoyladenosine-cAMP; A-kinase anchoring protein, AKAP; adenylate cyclase, AC; BSA, bovine serum albumin; COX-2, cyclooxygenase-2; Cx43, connexin-43; db-cAMP, dibutyryl cAMP; DMEM, Dulbecco's Modified Eagle's Medium; DPBS, Dulbecco's PBS; ECL, enhanced chemiluminescence; EIA, enzyme immunoassay; EP, PGE_2 receptor (protein); EPAC, exchange protein directly activated by cAMP; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GαS, G protein αS; HSP20, heat shock protein 20; KPSS, high potassium (60 mM KCl) physiological saline solution; LDS, lithium dodecyl sulphate; LTCC, L-type calcium channel; MIT, mean integral tension (area under the curve analysis); MLC20, myosin light chain 20; MYLP, myosin light chain phosphatase; OTR, oxytocin receptor; PDE, phosphodiesterase; PF 04418948, 1-(4-Fluorobenzoyl)-3-[[(6-methoxy-2-naphthalenyl)oxy]methyl]-3-azetidinecarboxylic acid; PGFS, PGF synthase; PKA, protein kinase A; PSS, physiological saline solution; PTGER, PGE_2 receptor (mRNA); PTGES, PGE synthase; PTGFR, $PGF_{2\alpha}$ receptor; PTL, preterm labour; PVDF, polyvinylidene fluoride; qPCR, quantitative PCR; RIIα, regulatory subunit RIIα; SERCA2, sarco/endo-plasmic reticulum calcium ATPase 2; SM-MHC, smooth muscle myosin heavy chain; TTBS, Tween-Tris-buffered saline.

INTRODUCTION

Over 15 million preterm births (≤ 37 weeks gestation) occur worldwide annually, with many resulting in neonatal death or life-long disability (March of Dimes, 2012). Preterm labour (PTL) is responsible for many of these premature births, and although acute tocolytic treatments can delay delivery they do not improve neonatal outcomes. Agents that increase intracellular cAMP content (hereon referred to as 'cAMP agonists') can inhibit myometrial contractions acutely. Indeed, β_2-adrenergic agonists, which work by increasing cAMP synthesis, were the mainstay of PTL therapy, but proved to be ineffective with long-term administration and are associated with life-threatening side effects. The short duration of β_2-agonist efficacy has been attributed to β_2-adrenoreceptor desensitisation, caused by a decline in receptor number (Michel *et al.*, 1989) and/or G protein uncoupling (Frambach *et al.*, 2005). However, interest persists in the use of drugs that increase cAMP to inhibit myometrial contractions (Mehats *et al.*, 2000; Croci *et al.*, 2007; Verli *et al.*, 2013) since this approach is successful in the management of other conditions associated with smooth muscle hyper-contractility, including asthma and pulmonary hypertension (Morgado *et al.*, 2012; Billington *et al.*, 2013). The focus has shifted to drugs that enhance cAMP levels independently of β_2-adrenoreceptor activation, such as cAMP-phosphodiesterase (cAMP-PDE) inhibitors (Mehats *et al.*, 2000; Verli *et al.*, 2013), as alternatives.

A body of evidence exists to support a role for cAMP in maintaining uterine quiescence during pregnancy (Yuan & Lopez Bernal, 2007). Many endogenous pro-pregnancy hormones increase cAMP levels; namely relaxin, human chorionic gonadotropin and calcitonin gene-related peptide. Key components that promote myometrial cAMP signalling are reduced upon labour onset, such as protein kinase A (PKA) regulatory subunit RIIα (MacDougall *et al.*, 2003), G protein α_S ($G\alpha_S$; (Europe-Finner *et al.*, 1994)) and heat shock protein 20 (HSP20, a PKA substrate; (Chan *et al.*, 2014)). The last of these, HSP20, has been used to demonstrate the importance of PKA-driven phosphorylation in promoting smooth muscle relaxation not only in the myometrium (Tyson *et al.*, 2008; Karolczak-Bayatti *et al.*, 2011), but also in the myocardium (Edwards *et al.*, 2012) and airway smooth muscle (Gerthoffer *et al.*, 2013). Additionally, myometrial levels of cAMP-specific phosphodiesterase 4 (PDE4) isoform B increase with preterm labour (Verli *et al.*, 2013). PKA has also been suggested to promote relaxation by repressing both intracellular calcium mobilisation, via inhibition of phosphatidylinositol turnover (Dodge & Sanborn, 1998), and myosin light chain 20 (MLC_{20}) phosphorylation, by repressing Rho signalling (Murthy *et al.*, 2003). These data, taken with

the ability of cAMP to acutely inhibit contractility, suggest that promoting myometrial cAMP signalling via PKA activation might be an effective approach to PTL prevention.

However, a previous publication suggested that maintaining relatively elevated levels of myometrial PKA activity, via prostacyclin administration, enhanced oxytocin-induced contractions by promoting expression of contractile apparatus proteins (Fetalvero *et al.*, 2008). Further, the exact role played by PKA in cAMP-induced myometrial relaxation is uncertain and recent data suggest that other cAMP effectors may be involved in smooth muscle relaxation (Roscioni *et al.*, 2011b).

In the present study, we investigated the role of PKA in acute cAMP-induced myometrial relaxation and assessed the impact of prolonged cAMP elevation on myometrial contractility. We tested the hypotheses that (i) acute cAMP-induced relaxation is directly proportional to total PKA activity; (ii) PKA activation alone can induce relaxation; and (iii) prolonged elevation of cAMP inhibits spontaneous and oxytocin-induced contractility.

MATERIALS AND METHODS

Ethical approval & tissue collection.

Myometrial biopsies from non-labouring pregnant women undergoing low-risk elective Caesarean section for term singleton pregnancies (37 + 5 to 41 + 4 weeks gestation; n = 87; Table 1) were collected in accordance with the Declaration of Helsinki guidelines, and with approval from the local research ethics committees for Guy's & St Thomas' Hospital (Ethics No. EC00/137) and Chelsea & Westminster Hospital (Ethics No. 10/H0801/45), London. Informed written consent was obtained from all women who participated. Biopsies were excised from the upper margin of incision made in the lower segment of the uterus and promptly immersed into sterile Dulbecco's PBS (DPBS) for storage at 2-4^{o}C until dissection for use within the same day.

Materials & reagents.

8-bromo-cAMP, dibutyryl cAMP (db-cAMP), forskolin and rolipram were purchased from Sigma-Aldrich (Dorset, UK). N^6-Benzoyladenosine-cAMP (6-Bnz-cAMP) was from Biolog (Bremen, Germany). Bay K8644 and 1-(4-Fluorobenzoyl)-3-[[(6-methoxy-2-naphthalenyl)oxy]methyl]-3-azetidinecarboxylic acid (PF 04418948) were from Tocris Bioscience (Bristol, UK). Syntocinon® was from Alliance Pharmaceuticals (Wiltshire, UK). Dulbecco's Modified Eagle's Medium (DMEM) and DPBS were from Sigma-Aldrich. Materials and reagents for SDS-PAGE and western blotting were from Life Technologies (Paisley, UK), Bio-Rad (Hertfordshire, UK) and Millipore (Hertfordshire, UK). Suppliers of primary antibodies are summarised in Table 2. All secondary antibodies were from New England BioLabs (Hertfordshire, UK). Primers were from Life Technologies. Suppliers of kits are stated elsewhere in the text. All other reagents were from Sigma-Aldrich, Merck Chemicals (Nottingham, UK) and Fisher Scientific (Loughborough, UK).

Isometric tension recording & data analysis for 1 hour acute response to cAMP agonists.

Isometric tension measurements were made using an 8-channel or 4-channel (Panlab; Barcelona, Spain) organ bath connected to ML870 PowerLab with MLT0201 transducers or 4SP PowerLab with MLT0210/D transducers (ADInstruments, Oxford, UK) respectively; samples subjected to identical experimental treatment protocols had their contractility output measured using the same organ bath apparatus. Longitudinal myometrial tissue strips used for assessing 1 hour 'acute' cAMP agonist treatment effects were dissected from biopsies in ice-cold DPBS and mounted into physiological saline solution (PSS, pH 7.4; composed of 119 mM NaCl, 4.7 mM KCl, 1.17 mM $MaSO_4$, 1.18 mM KH_2PO_4, 25 µM EDTA, 25 mM $NaHCO_3$, 6 mM glucose and 1.6 mM $CaCl_2$), maintained at 37^{o}C with 95 % O_2/5 % CO_2 aeration in organ bath chambers. Tension (29.4 mN) was applied to all tissues and PSS changed once every hour. After 1-1.5 hours of regular spontaneous contractions with a stable basal tension, cAMP agonist or vehicle (equivalent volume) was added with fresh PSS and incubated for 1 hour. Tissues were not used if they had not established stable contractions within 3 hours after applying tension. Each tissue strip treated with a cAMP agonist was paired with a vehicle-treated strip from the same biopsy.

At the end of treatment, PSS was changed to high potassium PSS (KPSS, pH 7.4; same composition as PSS except for concentrations of NaCl, 63.7 mM, and KCl, 60 mM) to induce maximal contractile response and confirm tissue viability. After 10 minutes, KPSS was replaced with PSS. After a further 10 minutes, tissues were cut off from their tied ends and into segments, which were weighed, snap frozen in liquid nitrogen and stored at -80^{o}C. All contractility data was analysed using LabChart 6 software (ADInstruments) to calculate mean integral tension values (MIT; area under the curve analysis for the sum of integrals for all contractions within the time period of interest, which was divided by its duration and mean cross-sectional area, the latter calculated from tissue strip mass and length (Herlihy & Murphy, 1973), for data normalisation). Ratios of MIT values for post-agonist (last 30 minutes prior to KPSS addition) relative to pre-agonist (last 30 minutes prior to cAMP agonist addition) treatment were calculated and converted to % MIT values.

Tissue culture, isometric tension recording & data analysis for 24 hours prolonged treatment with cAMP agonists.

Biopsies used for 24 hours pre-incubation to model 'prolonged' exposure to cAMP agonists were dissected under sterile conditions in cold DPBS and each attached to a glass weight of 0.6-0.7 g mass to maintain them under tension once suspended in serum-free DMEM supplemented with penicillin-streptomycin; each longitudinal tissue strip measured approximately 8 x 2 x 1 mm between the two points at which they were tied to thread. The choice of 0.6-0.7 g mass was based on experimental data from a previous study (Cordeaux *et al.*, 2010). Addition of cAMP agonists or vehicle controls to DMEM was done immediately before immersing tissues for incubation at 37^{o}C in a humidified atmosphere of 95 % air/5 % CO_2. Each tissue strip treated with a cAMP agonist was paired with a vehicle-treated strip from the same biopsy.

After 24 hours, tissues were either (i) cut from their tied ends and into segments to weigh and snap freeze in liquid nitrogen for -80^{o}C storage, or (ii) used for isometric tension recording experiments. For the former, untreated tissue pieces from matched biopsies were also snap frozen in liquid nitrogen to represent the state of the myometrium before 24 hours incubation, thus designated 't = 0' (i.e. time zero). We excluded db-cAMP from 24 hours incubation experiments because previous studies by others have suggested prolonged exposure to this

agonist can induce cAMP-independent cellular changes (Yusta *et al.*, 1988; Seternes *et al.*, 1999).

For isometric tension recordings, tissues, pre-incubated with cAMP agonists or vehicle, were mounted (after detaching their glass weights) into organ bath chambers and tension (29.4 mN) applied; PSS was refreshed every hour thereafter. At the third PSS change, Syntocinon® (1 nM; synthetic oxytocin), PF 04418948 (10 μM; PGE_2 receptor subtype 2 (EP2) antagonist), Bay K8644 (1 nM; L-type calcium channel (LTCC) agonist) or their vehicle controls was also added. For Bay K8644, each successive dose (or its vehicle) was added once every 25 minutes following a change of PSS each time. Subsequent incubations with KPSS, PSS washes, weighing, storage and data analysis were done in the same way as described for 1 hour acute response experiments. Absolute MIT values (in units of $mN/s/mm^2$) were used for final representation of the data (instead of % MIT) for spontaneous (last 50 minutes prior to Syntocinon® treatment) and oxytocin-induced (last 30 minute prior to KPSS addition) contractions.

Tissue homogenisation & PKA activity assay.

One frozen segment of each tissue strip was homogenised in ice-cold extraction buffer (25 mM Tris-HCl (pH 7.4), 0.5 mM EDTA and 0.5 mM EGTA), freshly supplemented with protease and phosphatase inhibitor cocktails as well as 10 mM β-mercaptoethanol, using a bead-based homogeniser (TissueLyser II homogeniser (Qiagen; Manchester, UK) or Precellys 24 homogeniser (Stretton Scientific; Derbyshire, UK)); samples with identical experimental treatment protocols were extracted using the same homogeniser. Homogenates were centrifuged at 15700 g at 4^oC for 5 minutes to isolate supernatants for assay, which were analysed for their relative protein concentrations using a Nanodrop ND-1000 spectrophotometer (LabTech; East Sussex, UK).

Manufacturer's protocol for the PepTag® Non-Radioactive cAMP-Dependent Protein Kinase Assay kit (Promega; Southampton, UK) was used with the following modifications for measuring basal PKA activity in tissue extracts: (i) cAMP stock supplied with the kit was not added, and (ii) 0.04 ng PKA substrate was used for each sample and control reaction. Total protein of homogenate supernatants (18 μg) was used for each sample and the reaction

duration was 45 minutes. Agarose gel electrophoresis was used to separate florescent non-phosphorylated and phosphorylated substrates, and gel images were captured using the Gel Logic 2200 Pro system (Carestream; Hertfordshire, UK) or G:BOX Chemical XL system (Syngene; Cambridge, UK); fluorescent gel bands were analysed by densitometry using ImageJ v1.46 software to obtain histogram-based total area pixel intensity values (National Institutes of Health; Maryland, USA), from which % phosphorylation of total assay substrate for each reaction was calculated.

Western blotting.

Protein samples from the same preparations of tissue extracts used for PKA assays were diluted in lithium dodecyl sulphate (LDS) sample buffer with 50 mM dithiothreitol for SDS-PAGE; precast Bis-Tris gels were used for all primary antibody targets except smooth muscle myosin heavy chain (SM-MHC), which was examined using precast Tris-glycine gels. The same amount of protein (5-15 µg) was loaded into gel wells for paired cAMP agonist and vehicle control samples, and for each primary antibody target. Western blotting was done using the XCell SureLock Mini-Cell & XCell II Blot Module system (Life Technologies) for Bis-Tris gels or the Trans-Blot Turbo Transfer system (Bio-Rad) for Tris-glycine gels; both western blotting systems used polyvinylidene fluoride (PVDF) membranes. Ponceau-S staining was used to visualise transfer efficiency and identify suitable areas for membrane cutting.

Membranes were blocked using 5 % w/v fat-free milk powder or bovine serum albumin (BSA) fraction V, both in Tween-Tris-buffered saline (TTBS), for un-phosphorylated and phosphorylated primary antibody targets, respectively. Duration of incubations with primary antibody (1-2 hours at room temperature or 10-16 hours at 4^{o}C) and enhanced chemiluminescence (ECL) reagent (1-10 minutes) was dependent on the primary antibody used. Secondary antibody incubation lasted for 1-2 hours at room temperature. The G:BOX Chemical XL system was used to capture images of chemiluminescent bands on PVDF membranes after incubation with ECL reagent. Densitometry analysis was done using ImageJ v1.46 software to obtain histogram-based total area pixel intensity values, and these values for proteins of interest were normalised to those for either glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β-tubulin from the same gel lane for each sample.

Tissue pulverisation & cAMP enzyme immunoassay.

One frozen segment of each tissue strip was pulverised into powder in liquid nitrogen using a stainless steel mortar and pestle. Extracts for cAMP quantification were prepared for enzyme immunoassay (EIA) using the DetectX Direct cAMP colorimetric EIA kit (Arbor Assays; Michigan, USA) following the manufacturer's protocol. A MRX II microplate reader and Revelation v4.2.2 software (Dynex Technologies; West Sussex, UK) were used for data acquisition.

Quantitative RT-PCR (qPCR).

A segment of each frozen tissue strip was homogenised using a TissueLyser II homogeniser (Qiagen), RNA was extracted using the TRIzol® Plus RNA Purification kit (Life Technologies) and cDNA conversion was done using the QuantiTect® Reverse Transcription kit (Qiagen). Primers were designed using Primer-BLAST (National Institutes of Health; Maryland, USA) and the sequences used are summarised in Table 3. HotStarTaq® Master Mix (Qiagen) and QIAquick® PCR Purification kits (Qiagen) were used for making qPCR standards for each set of primers using cDNA template of mRNA from untreated term non-labouring human myometrial tissue. Amplicon size and purity of qPCR standards were checked using DNA agarose gel electrophoresis. RNA and cDNA concentrations were quantified using a Nanodrop ND-1000 spectrophotometer. SYBR® Green PCR Master Mix (Life Technologies) and a Rota-Gene Q thermocycler (Qiagen) were used for qPCR. Cycling conditions used and method of data analysis with Rotor-Gene Q Software v2.0.3 (Qiagen) were as previously described (Lei *et al.*, 2011).

Statistical analysis.

Statistical analysis was performed using Prism v5.02 software (GraphPad; La Jolla, USA). Western blot data with $n \leq 5$ was log-transformed for parametric analysis. Treated and control groups were classed as unmatched for all contractility data to take into account tissue strip-to-strip variability of muscle fibre orientations (Crankshaw *et al.*, 2014). Statistical tests

used to identify significant differences ($p < 0.05$; indicated by one '*' at all figures irrespective of exact p values) were as follows: Mann-Whitney, Wilcoxon or Kruskal-Wallis test (with Dunn's post-hoc test for the latter) for isometric tension measurements; Wilcoxon or paired Student's t-test for cAMP EIA and PKA activity; paired Student's t-test for qPCR and western blot. Mean ± SEM values of fold differences calculated from cAMP agonist treatment relative to vehicle control are stated in the main text.

RESULTS

PKA-specific activation was not sufficient to mediate acute cAMP-driven relaxation.

Spontaneous contractions were reduced by cAMP analogues 8-bromo-cAMP (Figure 1A-Ca) and db-cAMP (Figure 1A-Cb) in a concentration-dependent manner. In contrast to this, the PKA-specific agonist, 6-Bnz-cAMP, did not reduce spontaneous myometrial contractions (Figure 1A-Cc) despite significantly stimulating PKA activity (6.8 ± 2.0 mean fold *versus* vehicle for 0.2 mM, $p = 0.0313$; Figure 1Bc). Rolipram (Figure 1A-Cd), a PDE4-specific inhibitor, and forskolin (Figure 1A-Ce), an adenylyl cyclase activator, inhibited spontaneous contractions, but, intriguingly, both were associated with relatively low increases in PKA activity (Figure 1Dd-e). To emphasise the weak relationship between relaxation and PKA activation, 0.1 mM 8-bromo-cAMP had no effect on spontaneous contractions ($p = 0.2188$; Figure 1A-Da) but did increase PKA activity to a similar extent as 1 µM forskolin (2.0 ± 0.8 and 2.0 ± 0.3 mean fold *versus* vehicle, respectively, and both $p = 0.0313$; Figure 1A-De).

Concentrations of cAMP in tissues used for *in vitro* tension measurements (Figure 1) were higher than vehicle control for 1 µM rolipram (2.5 ± 0.4 mean fold *versus* vehicle, $p = 0.0132$; Figure 2Aa), which was associated with a relatively small though nevertheless statistically significant increase in PKA activity (6.2 ± 3.8 mean fold *versus* vehicle, $p = 0.0313$; Figure 1Dd). Forskolin increased cAMP content, but not to a significant extent ($p = 0.1070$ (1 µM) and 0.2235 (10 µM); Figure 2Ab) even though PKA activity was increased, especially at 10 µM (5.4 ± 0.5 mean fold *versus* vehicle, $p = 0.0313$; Figure 1De), more robustly than rolipram. HSP20 Ser16-phosphorylation was increased by all cAMP agonists tested at their optimal pro-relaxation concentrations (Figure 2B); 6-Bnz-cAMP also increased HSP20 Ser16-phosphorylation despite failing to induce relaxation (Figure 1A-Cc). Double

bands were detected for both Ser16-phosphorylated and total HSP20 proteins, as reported previously (Karolczak-Bayatti *et al.*, 2011; Sin *et al.*, 2011); both were quantified and showed a similar pattern of change (Figure 2Bc, 2Bf). Levels of total HSP20 were unchanged in all cases except for a some reduction to its high molecular weight band form after 1 hour exposure to 1 mM db-cAMP (1.05 ± 0.2 mean fold *versus* vehicle, p = 0.0362; Figure 2Bd). For all subsequent western blots analysis, each phospho- and total HSP20 graph consists of values that equate to the sum of low and high molecular weight HSP20 bands detected together.

Prolonged treatment with cAMP agonists alone was able to maintain enhanced PKA activity.

Concentrations of cAMP agonists used for 24 hours treatment of tissues were those that gave ≥ 80 % acute reduction of MIT in our 1 hour experiments (Figure 1); with the exception of 6-Bnz-cAMP, for which 0.2 mM was used because it significantly activated PKA after acute treatment (Figure 1Dc). Myometrial tissues kept under tension during 24 hours treatment with 6-Bnz-cAMP or forskolin demonstrated higher PKA activity than vehicle controls (6.8 ± 1.8 and 3.8 ± 0.7 mean fold, respectively, and both p = 0.0313; Figure 3A) as well as, to statistically borderline extent, 8-bromo-cAMP (4.5 ± 1.5 mean fold *versus* vehicle, p = 0.0625; Figure 3Aa). Similar to our acute response data (Figure 1Dd), rolipram had no effect on PKA activity after 24 hours exposure (p = 0.1563; Figure 3Ab). HSP20 Ser16-phosphorylation (Figure 3Ba-b) was increased by forskolin, (4.0 ± 0.7 mean fold *versus* vehicle, p = 0.0015), rolipram (2.7 ± 0.7 mean fold *versus* vehicle, p = 0.0334) and, to borderline significance, 8-bromo-cAMP (2.1 ± 0.5 mean fold versus vehicle, p = 0.0606). In contrast to 1 hour treatment of spontaneously contracting tissues (Figure 2), 6-Bnz-cAMP had no effect on HSP20 Ser16-phosphorylation levels after 24 hours of exposure (Figure 3Ba). Total HSP20 protein levels were unchanged by cAMP agonists relative to their vehicle controls, but both H_2O and DMSO vehicle treatment for 24 hours were associated with greater content of total HSP20 protein in tissues when compared to time zero controls (1.6 ± 0.2, p = 0.0124, and 1.7 ± 0.1, p = 0.0008, mean fold *versus* time zero, respectively; Figure 3Bc-d).

Spontaneous contractility was maintained by prolonged treatment with 8-bromo-cAMP.

After assessing the ability of cAMP agonists to enhance PKA activity and/or HSP20 Ser16-phosphorylation following 24 hours exposure alone (Figure 3), a second set of tissues was subjected to the same treatments, but then used for isometric tension measurements to determine the impact of prolonged incubation with cAMP agonists on myometrial contractility *in vitro*; overall spontaneous contractile output appeared to be reduced in tissues cultured for 24 hours compared to those used in our 1 hour experiments (prior to acute cAMP agonist or vehicle treatment; Figure 1A). Pre-incubation with 6-Bnz-cAMP had no effect on spontaneous contractility when compared to its vehicle control. However, in tissues treated with 1 mM 8-bromo-cAMP (4.3 ± 1.2 mean fold *versus* vehicle, p = 0.0043), rolipram (4.4 ± 0.8 (1 µM) and 3.9 ± 1.2 (10 µM) mean fold *versus* vehicle; p = 0.0152 and 0.0260, respectively) and 10 µM forskolin (5.8 ± 1.5 mean fold *versus* vehicle, p = 0.0260), we observed greater spontaneous contractility (Figure 4Aa). In the case of 8-bromo-cAMP, this occurred despite elevated PKA activity as demonstrated in tissues from a third set of experiments (1.9 ± 0.3 mean fold *versus* vehicle, p = 0.0313; Figure 5A); Total calponin protein levels were increased in the same tissues (2.1 ± 0.3 mean fold *versus* vehicle, p = 0.0210; Figure 5B). HSP20 Ser16-phosphorylation levels were reduced in these tissues but not to a statistically significant extent (0.8 ± 0.4 mean fold *versus* vehicle, p = 0.2387; Figure 5C), while total HSP20 protein abundance was, on average, unchanged (Figure 5D). Cofilin Ser3-phosphorylation levels appeared, on average, unchanged (Figure 5E), while there was subtle, though not statistically significant, reduction of total cofilin protein abundance after prolonged exposure to 8-bromo-cAMP (0.9 ± 0.1 mean fold *versus* vehicle, p = 0.3509; Figure 5F).

Oxytocin (1 nM Syntocinon®) stimulation was able to induce robust rhythmic contractions in both vehicle and cAMP agonist treated tissues, to similar extents, after 24 hours despite low spontaneous contractile output from the former (Figure 4Ab). The oxytocin-induced MIT values were not significantly greater in 8-bromo-cAMP pre-incubated tissues compared to vehicle control (Figure 4Ab-c). Interestingly, PKA activity measured after oxytocin stimulation (Figure 4B) was higher for tissues pre-incubated for 24 hours with 8-bromo-cAMP (2.0 ± 0.3 mean fold *versus* vehicle, p = 0.0180) and 6-Bnz-cAMP (1.5 ± 0.1 mean fold *versus* vehicle, p = 0.0229).

We found no significant changes in protein levels for connexin-43 (Cx43), oxytocin receptor (OTR) and sarco/endo-plasmic reticulum calcium ATPase 2 (SERCA2) (data not shown). We also found no difference in SM-MHC or calponin (data not shown) levels after oxytocin stimulation. Levels of cyclic AMP-related proteins, PDE4B, PKA RIIα and $G\alpha_S$ were also not different when compared to their vehicle controls (data not shown). A lack of difference in abundance of the same contraction-associated and cAMP signalling proteins was also noted for tissues measured only for their spontaneous contractile output after 24 hours pre-incubation with 8-bromo-cAMP compared to vehicle control (data not shown). Interestingly, 8-bromo-cAMP and rolipram pre-incubated tissues contained HSP20 Ser16-phosphorylation levels that were subtly lower than vehicle controls after oxytocin treatment (Figure 6Aa), which followed the same trend as observed for 8-bromo-cAMP pre-incubated strips measured only for their spontaneous contractions (Figure 5C). In addition to this, total HSP20 protein levels were found to be significantly reduced following oxytocin stimulation of tissues pre-incubated with 8-bromo-cAMP (0.7 ± 0.04 mean fold *versus* vehicle, p = 0.0100; Figure 6Ab). Total cofilin protein abundance was also slightly though significantly reduced (0.6 ± 0.1 mean fold *versus* vehicle, p = 0.0189; Figure 6B).

The effect of cAMP agonists on the prostaglandin system.

Cyclooxygenase-2 (COX-2) protein levels were not affected by 24 hours incubation with cAMP agonists (Figure 7A). The mRNA expression levels for prostaglandin receptors and synthases, namely PGE_2 receptor subtype 3 (PTGER3), PGE synthase types 1-3 (PTGES, PTGES2, PTGES3), $PGF_{2\alpha}$ receptor variant 1 (PTGFR v1) and PGF synthase (PGFS), were also unaffected by pre-treatment with 8-bromo-cAMP compared to vehicle control (data not shown); PGE_2 receptor subtypes 1 (PTGER1) and 4 (PTGER4), along with $PGF_{2\alpha}$ receptor variant 2 (PTGFR v2), mRNAs were undetectable. Whereas PGE_2 receptor subtype 2 (PTGER2) mRNA levels were significantly greater in 8-bromo-cAMP pre-incubated strips (1.7 ± 0.1 mean fold versus vehicle, p = 0.0030), and assessment of tissues from equivalent experiments with the other cAMP agonists (Figure 4) showed the same, though to a slightly lesser extent, for 10 μM rolipram (1.6 ± 0.2 mean fold versus vehicle, p = 0.0055; Figure 7B). However, EP2 receptor antagonist, PF 04418948, treatment on a separate set of 8-bromo-cAMP pre-incubated tissues during isometric tension measurements, in the absence of

oxytocin stimulation, showed the pro-relaxation effect of EP2 receptors was in fact functionally maintained to the same extent as vehicle pre-incubated tissues (Figure 7C).

Myometrial contractions in the presence of a LTCC agonist were unaffected after 24 hours treatment with 8-bromo-cAMP.

LTCCs provide the main route of extracellular calcium entry (Tribe, 2001), are regulated by PKA (Chik *et al.*, 1996; Zhong *et al.*, 1999) and their activity is increased in the myometrium of labouring women (Longo *et al.*, 2003). We used a non-cumulative dosing protocol with the LTCC agonist, Bay K8644, to identify any differences in the amount of contractile force generated from incrementally increasing the population of LTCCs in their open state after prolonged 8-bromo-cAMP incubation. Although tissues treated with 8-bromo-cAMP for 24 hours tended to produce greater increases in contractile output after successive doses of Bay K8644 per unit of time, the MIT values for each dose were not significantly different to those of vehicle control (Figure 8).

DISCUSSION

In the current study, we sought to understand the role of PKA in acute cAMP-induced myometrial relaxation and to study the impact of prolonged maintenance of elevated cAMP levels on myometrial contractility. The latter aimed to assess the use of agents able to enhance cAMP levels without cell surface receptor activation for the prevention of preterm labour. We found that it was possible to significantly increase PKA activity without affecting spontaneous or induced myometrial contractility. This was unexpected given that it has long been held that cAMP acts via PKA to promote myometrial relaxation (Anwer *et al.*, 1990; MacDougall *et al.*, 2003; Tyson *et al.*, 2008). Conversely, the prolonged maintenance of cAMP levels and PKA activity did not reduce spontaneous or oxytocin-induced myometrial contractions but, most notably in the case of 8-bromo-cAMP, actually resulted in greater spontaneous contractile output.

The contribution of PKA to acute cAMP-driven myometrial relaxation.

The pro-relaxation concentrations we determined for 8-bromo-cAMP, rolipram and forskolin, by applying these cAMP agonists to spontaneously contracting tissues as single doses for 1 hour incubation, matched well with previous studies that used the same cAMP agonists to observe cumulative dosing effects (Mehats *et al.*, 2000; Slater *et al.*, 2006; Tyson *et al.*, 2008). The ability of cAMP to acutely inhibit myometrial contractions is often attributed to PKA activation (Tyson *et al.*, 2008; Karolczak-Bayatti *et al.*, 2011). Indeed our experiments showed that tissues treated with pro-relaxation concentrations of 8-bromo-cAMP and db-cAMP contained significantly higher levels of PKA activity than those treated with vehicle control.

However, the findings of our present study also demonstrated acute PKA activation alone does not mediate relaxation in human myometrial tissue despite being able to promote HSP20 Ser16-phosphorylation, as shown by (i) lack of pro-relaxation effects from a PKA-specific agonist, 6-Bnz-cAMP, and (ii) lack of correlation between PKA activity and degree of relaxation achieved with different cAMP agonists. The latter was clearly demonstrated by rolipram's ability to induce myometrial relaxation without significantly increasing PKA activity, as well as by forskolin's ability to induce profound relaxation with modest PKA activation necessary. These data suggest other cAMP signalling components may be necessary for promoting myometrial relaxation, such as PDE4. Indeed, in cardiomyocytes, PDE4 binds to HSP20 to prevent its phosphorylation by PKA (Sin *et al.*, 2011); HSP20 activation, which leads to muscle relaxation by promoting actin depolymerisation via cofilin (Karolczak-Bayatti *et al.*, 2011), occurs when cAMP levels are increased in close physical proximity to PDE4, by stimulating its dissociation from HSP20. If a similar sequence of events occurs in myometrial cells, it suggests that cAMP analogues activate PKA, but do not induce relaxation at low concentrations due to their indiscriminate intracellular distribution. Indeed, both 8-bromo-cAMP and db-cAMP are poor PDE substrates, compared to endogenous cAMP, but are able to saturate PDE4 active sites when present in high concentrations (Schultz *et al.*, 1994; Xu *et al.*, 2004), perhaps explaining why their ability to increase PKA activity preceded their pro-relaxation effects. However, 6-Bnz-cAMP also has a low affinity for PDE4 (Poppe *et al.*, 2008) while still able to promote HSP20 Ser16-phosphorylation without inducing relaxation, suggesting a yet to be elucidated distinction in PKA-independent aspects of cAMP-driven muscle relaxation between the heart and myometrium.

Our detection of low PKA activity despite measurable increases in cAMP levels following rolipram treatment, as well as high PKA activity despite less detectable levels of cAMP elevation following forskolin treatment, could be attributed to the activation status of adenylate cyclases (ACs) playing more of an important role towards the amount of PKA activity achieved than the amount of cAMP they synthesise in total. Microdomain cAMP signalling is also an important potential contributor, especially when considering that the present study measured PKA activity and intracellular cAMP concentrations using whole tissue extracts, and thus any intracellular localised elevations of both would have been diluted once tissues were homogenised. PDE4 inhibition by rolipram would reduce the hydrolysis of cAMP in myometrial cells in a resting state, as AC activity is unlikely to be enhanced by rolipram treatment. On the other hand, forskolin treatment mimics $G\alpha_s$-coupled GPCR activation by directly enhancing AC activity. Thus, we could speculate that the active conformation of ACs sensitise PKA activity, via mechanisms such as protein-protein interactions via lipid rafts (Crossthwaite *et al.*, 2005) and A-kinase anchoring proteins (AKAPs), to minimise the amount of cAMP necessary for its enhancement. In the latter case, it is also well-established that ACs and PKA are often held in close proximity to each other via AKAP79, a protein that is known to be expressed in human myometrial tissue (Ku *et al.*, 2005), to form efficient signalling complexes (Cooper & Tabbasum, 2014).

If we also speculate that cAMP-hydrolysing PDEs control cAMP levels in specific intracellular microdomains, such as around the local vicinity of ACs and PKA at the plasma membrane of intact cells, and prevent its diffusion via region-restricted hydrolysis, which would explain why the total amount of cAMP would appear low when measured from whole tissue extract following forskolin treatment. Whereas PDE4 inhibition by rolipram would cause loss of localised cAMP elevation (Houslay, 2010), which could increase total cellular levels of cAMP sufficiently to make it detectable via enzyme immunoassays, after acute treatment, but at the same time not appear to significantly increase PKA activity levels due to the absence of necessary interactions between ACs and PKA. The apparent lower potency of 8-bromo-cAMP, db-cAMP and 6-Bnz-cAMP towards mediating myometrial relaxation, relative to PKA activity enhancement, may also be attributed to their lack of effect on AC-related interactions, in addition to their low binding affinity for PDE4. The importance of microdomain cAMP signalling is becoming increasingly well-defined in both cardiovascular (Zaccolo & Pozzan, 2002; Sprenger *et al.*, 2015) and airway smooth muscle (Bogard *et al.*,

2011; Patel *et al.*, 2015), but is relatively less explored in human myometrial smooth muscle (Dodge *et al.*, 1999; Mehats *et al.*, 2000; MacDougall *et al.*, 2003).

Previously published enzyme kinetics data gathered using purified proteins (Poppe *et al.*, 2008) have shown 8-bromo-cAMP and 6-Bnz-cAMP are capable of activating protein kinase G (PKG), but their half-maximal activation constants are >60-fold higher than for PKA. Nevertheless, we cannot discount the possibility that high concentrations of these agonists, such as those required for near-complete cessation of spontaneous contractions at acute response experiments, could activate the cGMP pathway. Indeed, it is known that HSP20 can be phosphorylated at its Ser16 residue by PKG (Beall *et al.*, 1997). Protein structure analysis has previously shown forskolin is unlikely to be able to activate cGMP synthesis, at least via soluble guanylate cyclase activation (Allerston *et al.*, 2013), and biochemical studies have shown forskolin itself is unable to mediate cell-based cGMP elevation (Watson *et al.*, 1990). Similar studies have also been done to show rolipram is specific to cAMP-hydrolysing PDEs (Delpy *et al.*, 1996; Herman *et al.*, 2000). Thus forskolin and rolipram treatment are likely to be associated with greater specificity for the activation of signalling via cAMP rather than cGMP when compared to the cAMP structural analogues, which may partly explain why they differ in their efficiencies for promoting myometrial relaxation during short-term exposure.

The impact of prolonged cAMP agonist exposure on myometrial spontaneous contractility and PKA activity.

Spontaneous contractility of tissues after 24 hours incubation was notably greater after incubation with 8-bromo-cAMP and, to a lesser though nevertheless significant extent, with rolipram and forskolin. Tissues pre-incubated with 8-bromo-cAMP also demonstrated increased calponin, as well as hinted towards reduced levels of both HSP20 Ser16-phosphorylation and total cofilin protein. Calponin has been shown to increase myometrial contractility in mice (Li *et al.*, 2012), cofilin promotes actin depolymerisation when not phosphorylated at its Ser3 residue and HSP20 Ser16-phosphorylation promotes activation of cofilin (Karolczak-Bayatti *et al.*, 2011; Hocking *et al.*, 2013). Consequently, these protein-level changes observed after prolonged exposure to 8-bromo-cAMP may have contributed to the relatively greater spontaneous myometrial contractility we observed. The reduction in HSP20 Ser16-phosphorylation detected after contractility measurements with 8-bromo-

cAMP pre-incubated tissues occurred despite elevated PKA activity and thus may be attributed to increased phosphatase activity. Indeed, phosphatases have been suggested to play an important role in the propagation of rhythmic contractions via tightly controlling contractile apparatus-regulating protein phosphorylation (Butler *et al.*, 2013), although this aspect of myometrial function has not been well-explored beyond examining myosin light chain phosphatase (MYLP, a protein phosphatase 1 holoenzyme; (Hudson *et al.*, 2012). Calcineurin, also known as calcium-activated protein phosphatase 2B, dephosphorylates cofilin to control tracheal smooth muscle contractility (Zhao *et al.*, 2008) and may play a similar role in the myometrium (Ayres *et al.*, 2003). Another possible explanation for the decline in HSP20 Ser16-phosphorylation may be attributed to 8-bromo-cAMP being able to activate PKG when used at high concentrations (as discussed above) and thus negative feedback on HSP20 Ser16-phosphorylation may have originated from the prolonged activation of PKG instead of altered PKA activity; the same possibility could also apply to the loss of HSP20 Ser16-phosphorylation following prolonged exposure to 6-Bnz-cAMP. The increase in total HSP20 protein levels following 24 hours in tissue culture relative to 'time zero' control samples, irrespective of cAMP agonists or vehicle treatment conditions, would appear to be consistent with the decline in spontaneous contractile output of all except 8-bromo-cAMP pre-incubated tissues, and most likely attributed to the *in vitro* culture conditions not being an exact replication of the *in utero* environment.

Despite a profound enhancement of spontaneous contractile output following prolonged exposure to 8-bromo-cAMP, we found that alterations to levels of several contraction-associated and cAMP signalling proteins often recognised as markers of labour in the myometrium were not necessary for this observation made in the present study. This included COX-2 protein, which was in conflict with our previous findings from cultured myometrial cells that showed prolonged treatment with cAMP agonists can enhance its expression levels (Chen *et al.*, 2012). This discrepancy could be attributed to a difference in complexity of the extracellular environment that exists between passaged myometrial cells grown in monolayer formation and tissue-based myometrial cells bound to a three-dimensional extracellular matrix alongside other cell types, which may cause differences in response to cAMP agonists due to, for example, greater buffering effects experienced by the latter. We detected no differences in the expression of several mRNA transcripts for receptors and synthetic enzymes of the prostaglandin pathway (Astle *et al.*, 2005; Astle *et al.*, 2007; Hay *et al.*, 2010)

in 8-bromo-cAMP pre-incubated tissues (after oxytocin exposure), with the exception of PTGER2 mRNA that encodes EP2 receptors. Interestingly, EP2 receptors can change their G protein coupling from pro-relaxation $G\alpha_s$ to pro-contractile $G\alpha_q$ upon labour onset in myometrial cells (Kandola *et al.*, 2014). We hypothesised prolonged exposure to 8-bromo-cAMP promoted spontaneous contractions by up-regulating $G\alpha_q$-coupled EP2 receptor activity, and thus expected a decline in spontaneous contractions from 8-bromo-cAMP pre-incubated tissues when acutely treated with EP2 receptor-specific antagonist, PF 04418948, but we found this to not be the case.

The 8-bromo-cAMP-induced increase in intracellular cAMP activity is relatively PDE-resistant and may actually drive the pro-contractile changes we observed as part of a negative feedback response. Indeed, such a response has been reported for primary myometrial cells, which increase their PDE4 enzyme activity (Mehats *et al.*, 1999) and decrease their $G\alpha_S$ gene promoter activity (Phillips *et al.*, 2002) after exposure to forskolin for 5 hours or more. In our experiments, forskolin and rolipram enhanced spontaneous contractions to a relatively lesser, but nevertheless significant, extent than 8-bromo-cAMP after 24 hours exposure, which may be attributed to the more hydrolysable nature of endogenous cAMP compared to the structural analogue form of 8-bromo-cAMP. This apparent time-dependent role reversal in cAMP control of myometrial activity, from pro-relaxation to pro-contractile, may contribute to the β_2-agonist tolerance effect seen from their use as tocolytics by modulating signalling processes beyond the cell surface receptors. Thus it may not be possible to by-pass desensitization of cAMP-mediated uterine relaxation associated with β_2-adrenoreceptor activation via use of receptor-independent cAMP agonists alone. Glucocorticoids prevent β_2-adrenoceptor agonists-induced desensitization (Mak *et al.*, 1995) and it is possible that the combination of progesterone and a cAMP agonist may have a similar effect in the myometrium. Indeed, we have previously published work to suggest cAMP agonists could be combined with progesterone to improve efficacy of the latter in reducing myometrial inflammation (Chen *et al.*, 2014) and are currently assessing the possibility that this combination could also better reduce contractile output of myometrial tissue than either agent alone.

Since the PKA-specific activator we assessed had no effect on spontaneous contractility, it is possible that the effects of 8-bromo-cAMP are mediated through a cAMP-dependent interaction with proteins other than PKA. The lack of change in both EP2 receptor and LTCC

function after 8-bromo-cAMP pre-incubation suggests the activity of these proteins did not contribute to the greater spontaneous contractile output that resulted from this treatment. Another potential candidate is EPAC, which binds directly to cAMP with an affinity that closely matches that of PKA (Dao *et al.*, 2006), but it is able to act independently of PKA activity in vascular and airway smooth muscle cells (Kassel *et al.*, 2008; Purves *et al.*, 2009; Eid, 2012). Its function in myometrial tissue is unknown, but EPAC and PKA have been shown to work together to inhibit smooth muscle proliferation and enhance contractility in airway smooth muscle cells (Hewer *et al.*, 2011; Roscioni *et al.*, 2011a).

The impact of prolonged cAMP agonist exposure on myometrial contractility and PKA activity after oxytocin stimulation.

All cAMP agonists failed to prevent oxytocin-induced contractions after prolonged exposure, despite detectable enhancement of PKA activity and/or HSP20 Ser16-phosphorylation (to a similar extent as observed with acute relaxation from 1 hour of treatment) after 24 hours of pre-incubation alone. It has been previously reported that 48 hours of cAMP elevation can enhance both SM-MHC and calponin expression in myometrial tissue, as well as increase oxytocin-induced contractile output (Fetalvero *et al.*, 2008). Connexin-43 (Cx43), oxytocin receptor (OTR) and sarco/endo-plasmic reticulum calcium ATPase 2 (SERCA2) have all been previously identified as contraction-associated proteins in the myometrium (Kimura *et al.*, 1996; Sparey *et al.*, 1999; Tribe *et al.*, 2000). However, our oxytocin-treated tissues showed no significant change in the levels of these proteins associated with the contractile apparatus and cAMP signalling. Instead, where we detected subtle decreases in total cofilin and HSP20 Ser16-phosphorylation in tissues that demonstrated enhanced spontaneous contractile output following pre-incubation with 8-bromo-cAMP, both also showed a decline in abundance following oxytocin stimulation. Together, our data from these experiments suggest prolonged activation of cAMP signalling does not result in lasting changes to the abundance of key proteins previously suggested to characterise myometrial labour-induced changes, both in the event of oxytocin-mediated augmentation of contractions and enhanced spontaneous contractions caused by prolonged exposure to 8-bromo-cAMP.

Summary.

In conclusion, the relationship between PKA activity and contractile output within the human myometrium is not a direct one. Other components of the cAMP signalling pathway are likely to make greater direct contributions towards mediating its well-recognised acute pro-relaxation effects, such as ACs and PDEs, with the control of intracellular microdomain signalling also playing a key role. Our novel observation of 8-bromo-cAMP's ability to promote spontaneous contractions, after prolonged exposure, demonstrates the potential importance of duration and spatial distribution of cAMP signalling in the regulation of myometrial contractility (Figure 9-10); it is unlikely to be related exclusively to PKA activation and potentially involves other cAMP-sensitive proteins or even alternative pathways, especially those of influence to the dynamics of actin filament function. Our findings suggest that prophylactic use of receptor-independent cAMP agonists alone, with intent to ubiquitously enhance myometrial cAMP accumulation and PKA activity, is unlikely to prevent the onset of preterm labour in high-risk women. However, cAMP modulation could remain a potential therapeutic target, if the need to control the intensity of cAMP augmentation necessary to maintain its pro-relaxation effects and a strategy to avoid negative feedback is considered. This may require combining its beneficial actions with those of tocolytic agents that act on alternative pathways to provide the balance required to achieve myometrial quiescence.

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ADDITIONAL INFORMATION

Competing interests

The authors have no conflicts of interest to declare.

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Author contributions

PFL, RMT and MRJ designed the experiments. PFL did the experiments and analysed the data. PFL, RMT and MRJ wrote the manuscript.

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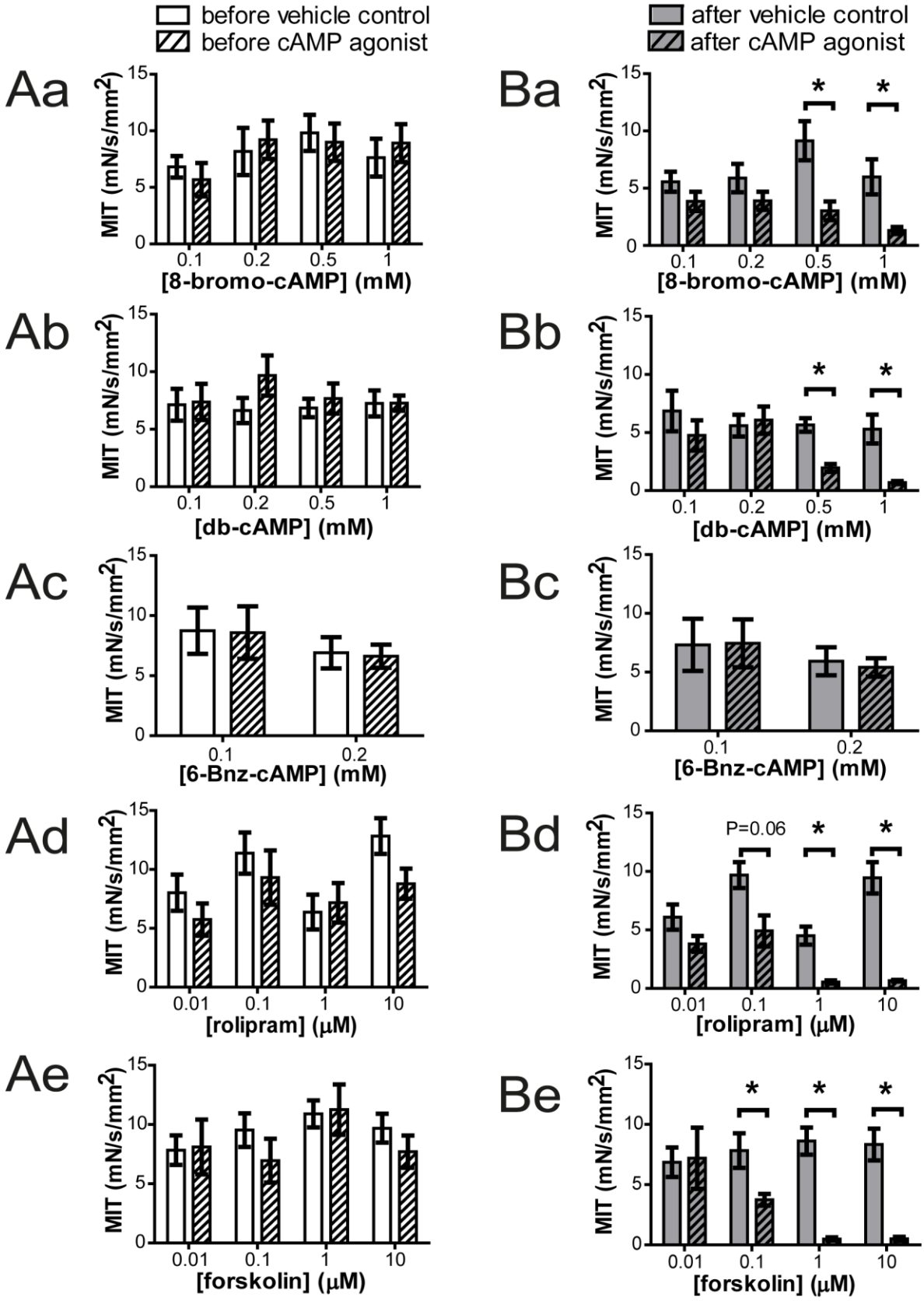
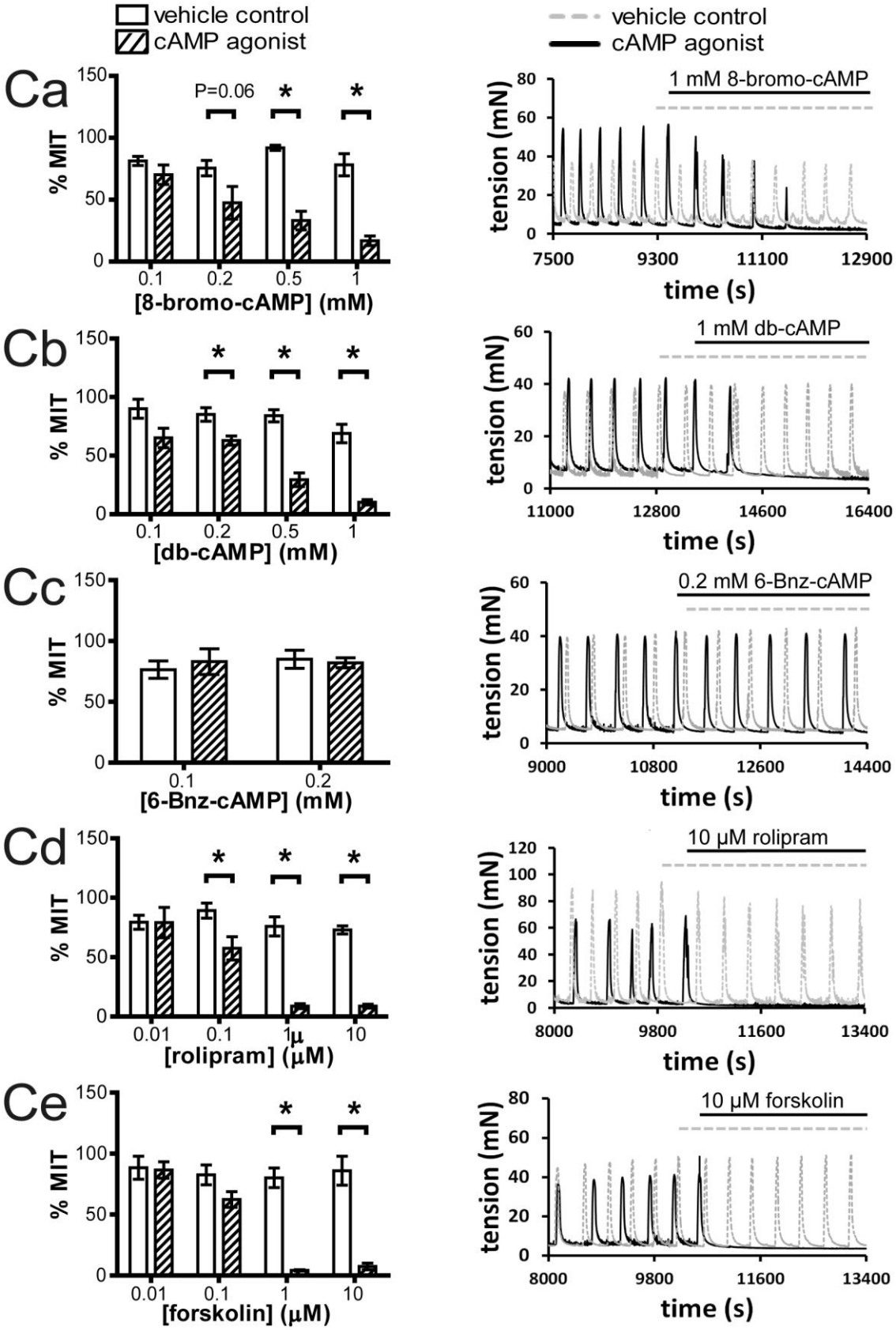
FIGURE LEGENDS

Figure 1. Disparity in effectiveness of cAMP agonists (1 hour exposure) to reduce spontaneous contractility of pregnant human myometrial tissue compared to PKA activation. Absolute mean integral tension (MIT) values for 30-minute period immediately prior to **(A)** addition of treatment and **(B)** end of 1 hour incubation with cAMP agonist or vehicle control, which were used to calculate **(C)** % MIT of spontaneously contracting human myometrial tissues *in vitro* for different concentrations of **(a)** 8-bromo-cAMP, **(b)** db-cAMP, **(c)** 6-Bnz-cAMP, **(d)** rolipram and **(e)** forskolin; representative traces of tension changes during contractions before and, as indicated by horizontal lines, during cAMP agonist or vehicle control treatment are also shown. **(D)** Protein kinase A (PKA) activity content of homogenate extracts from the same tissues, calculated as % phosphorylation of total assay substrate, alongside representative gel images of phosphorylated ('+') and non-phosphorylated ('-') PKA activity assay substrate after agarose gel electrophoresis for pair-matched vehicle control ('C') and cAMP agonist ('T') treated samples. All data presented as mean ± SEM (n = 6); statistically significant differences between cAMP agonist and vehicle control treatment denoted by * ($p < 0.05$) and 'P=0.06' for borderline significance ($p = 0.0649$ for 0.1 µM rolipram *versus* vehicle control in (Bd); $p = 0.0649$ for 0.2 mM 8-bromo-cAMP *versus* vehicle control in (Ca)).





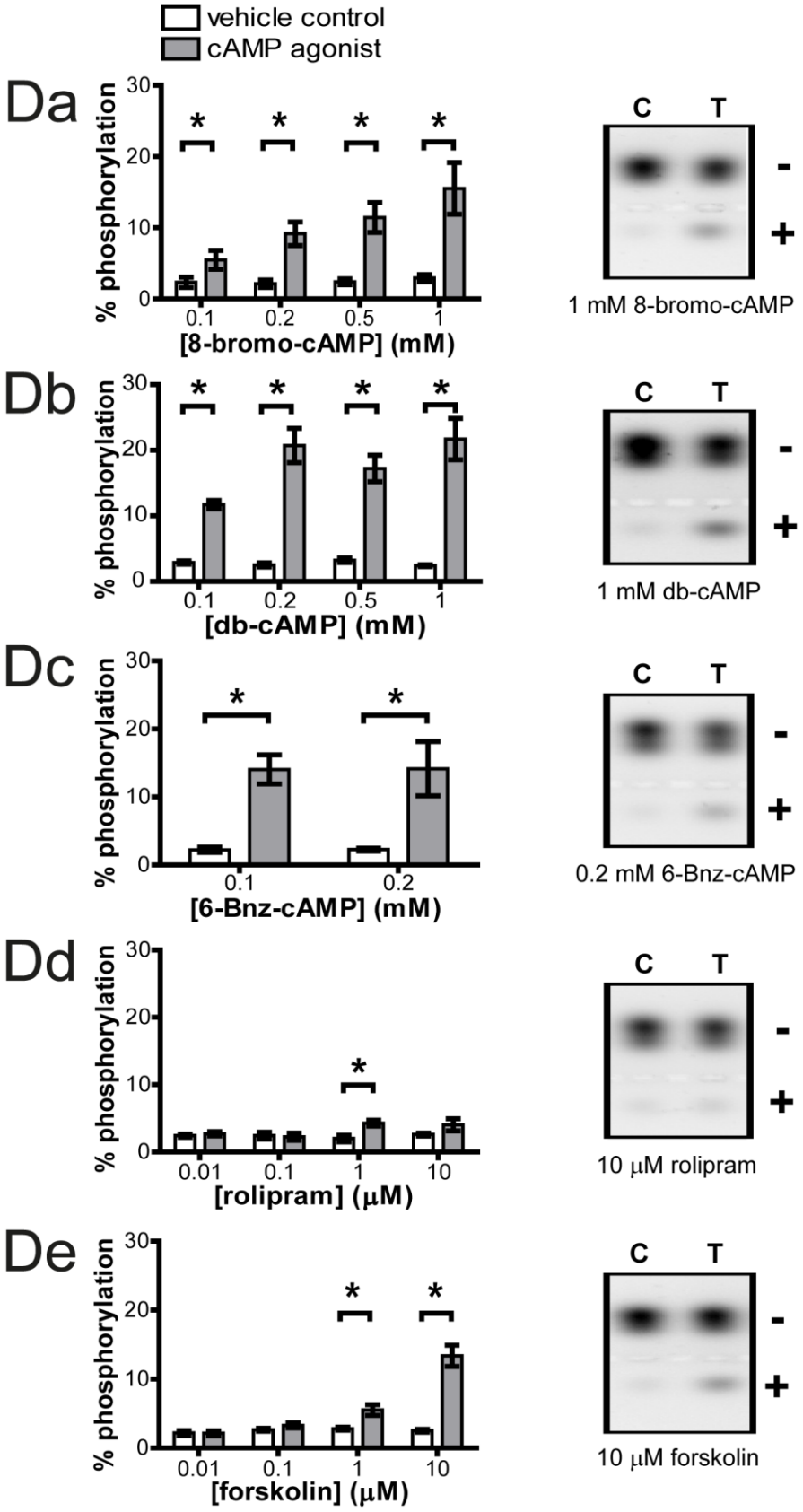
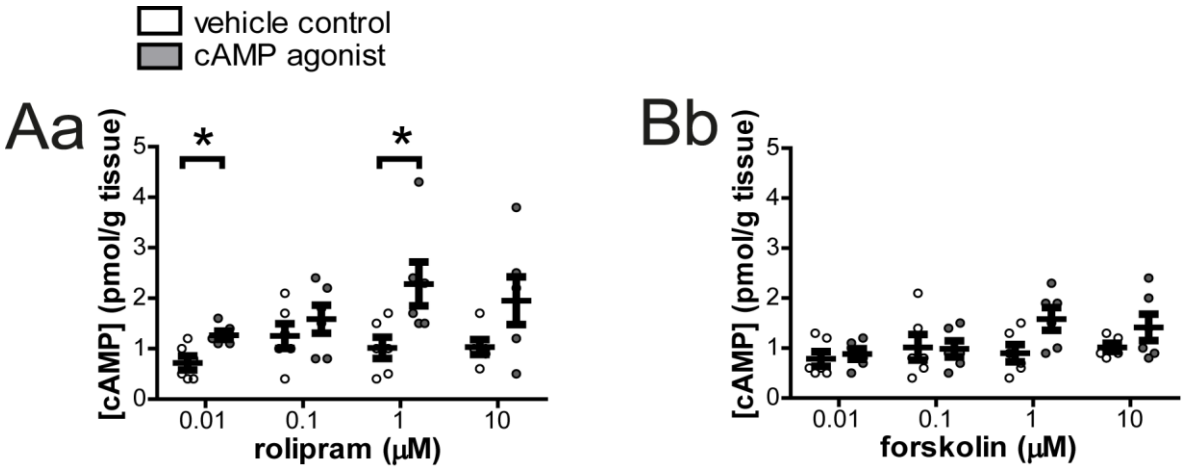


Figure 2. Intracellular cAMP concentrations and HSP20 Ser16-phosphorylation levels did not correlate with intensity of relaxation and PKA activity achieved after incubating spontaneously contracting pregnant human myometrial tissue with cAMP agonists for 1 hour. Tissue cAMP content quantified by enzyme immunoassay (EIA) for spontaneously contracting tissues treated for 1 hour with either **(Aa)** rolipram or **(Ab)** forskolin (same tissues as represented in Figure 1) presented as mean ± SEM (n = 6). Phosphorylated and total heat shock protein 20 (HSP20) levels for **(Ba, Bd)** ~19 kDa ('high') and **(Bb, Be)** ~17 kDa ('low') molecular weight protein bands, detected by western blotting with the same protein extracts used for protein kinase A (PKA) activity assays shown in Figure 1, presented as mean ± SEM (n = 4-5). Statistically significant differences are denoted by * ($p < 0.05$) for both (A) and (B). Representative western blot images for **(Bc)** Ser16-phosphorylated ('Phospho-HSP20') and **(Bf)** total HSP20 protein are shown below their corresponding graphs, alongside their matched glyceraldehyde 3-phosphate dehydrogenase (GAPDH) loading controls; positions of 'high' (thick arrow) and 'low' (thin arrow) HSP20 bands indicated next to each immunoblot image of pair-matched vehicle control ('C') and cAMP agonist ('T') treated samples.



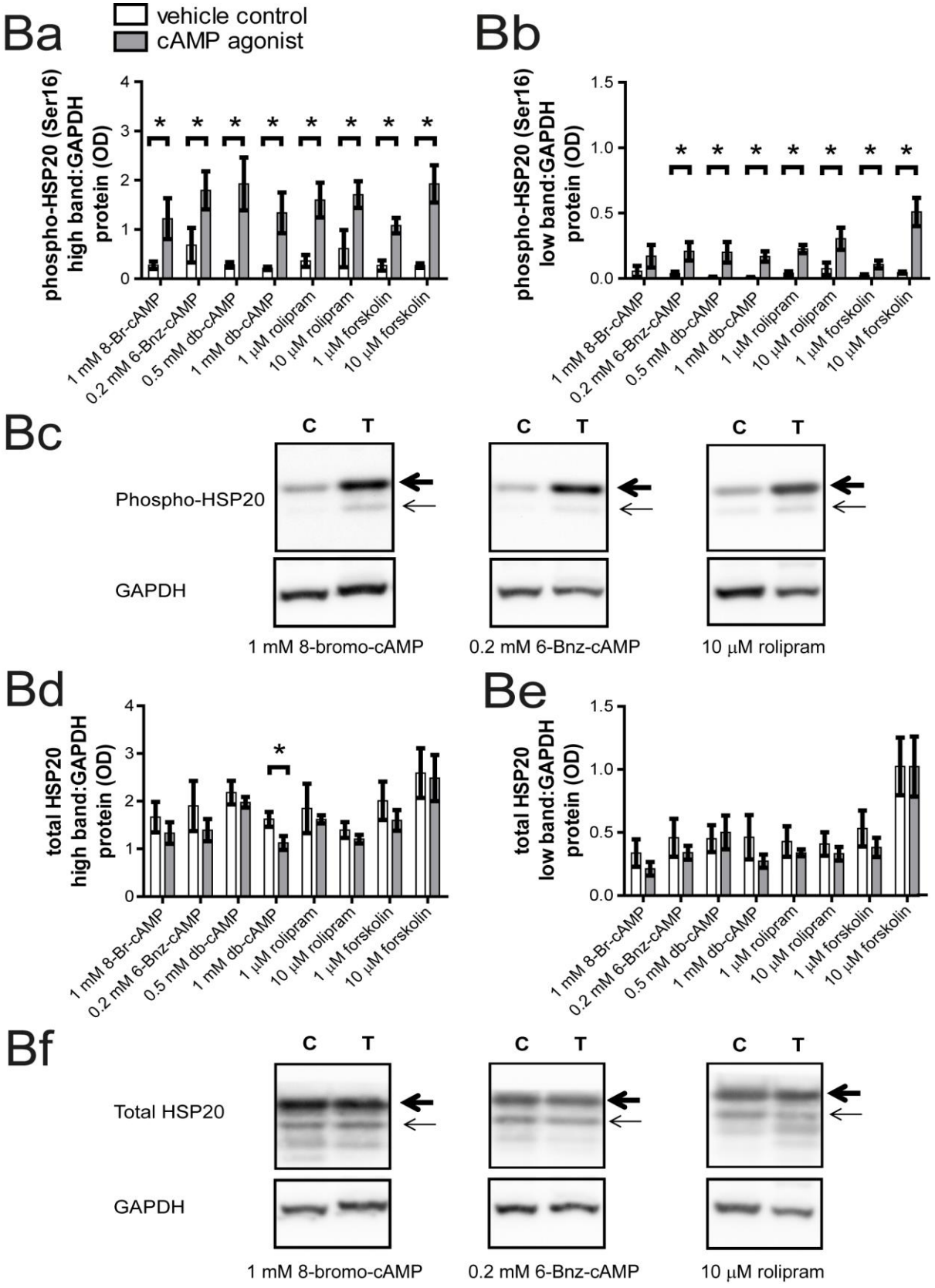
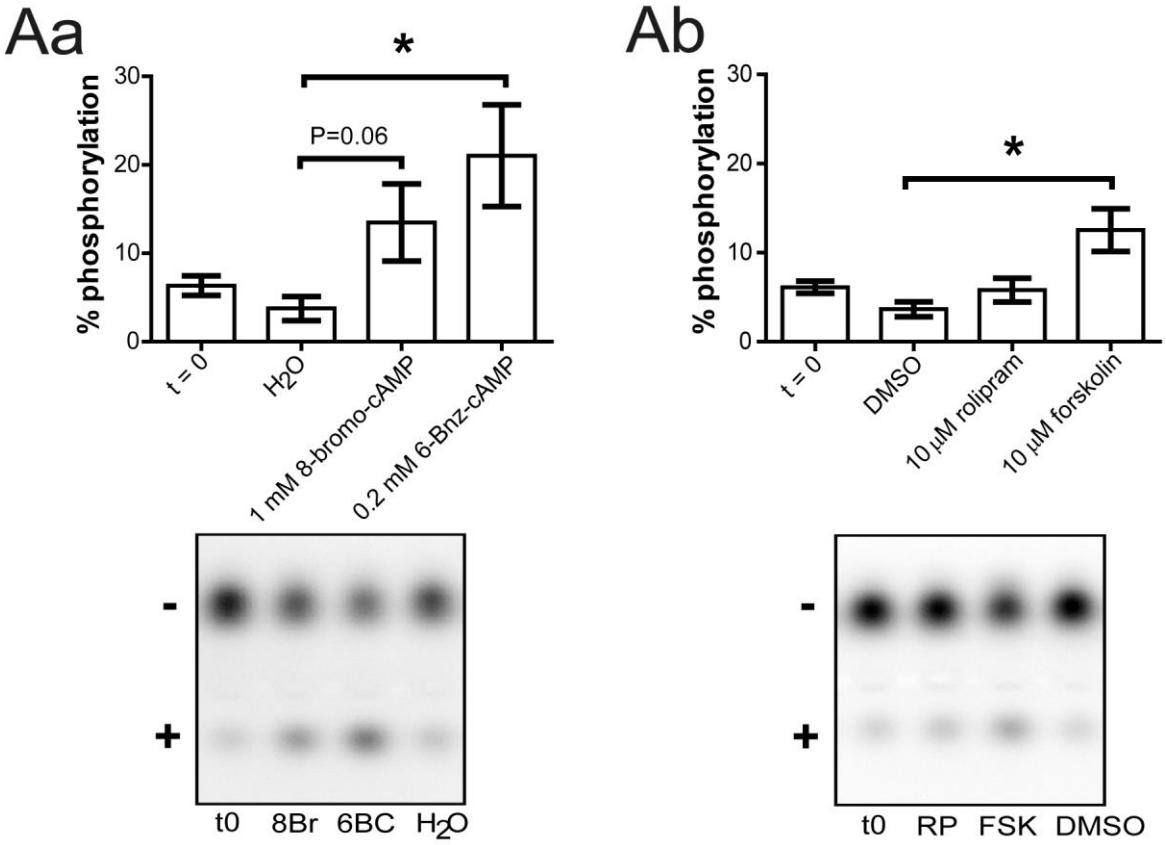


Figure 3. Treatment of pregnant human myometrial tissue maintained under tension for 24 hours with cAMP agonists enhanced PKA activity and HSP20 Ser16-phosphorylation to differing extents. Protein kinase A (PKA) activity measured from protein extracts of tissues incubated with **(Aa)** H_2O- (8-bromo-cAMP and 6-Bnz-cAMP) or **(Ab)** DMSO- soluble (rolipram and forskolin) cAMP agonists for 24 hours; untreated tissue samples snap frozen in liquid nitrogen at time of initiating treatment at tissue strips, dissected from matched biopsies, ('time zero', t = 0) were also assessed. PKA activity data presented as mean ± SEM (n = 6). Representative gel images of phosphorylated ('+') and non-phosphorylated ('-') PKA assay substrate shown below corresponding graphs, with labels for time zero ('t0'), 8-bromo-cAMP ('8Br'), 6-Bnz-cAMP ('6BC'), H_2O vehicle ('H_2O'), rolipram ('RP'), forskolin ('FSK') and DMSO vehicle ('DMSO'). Ser16-phosphorylated and total heat shock protein 20 (HSP20) levels for **(Ba, Bc)** H_2O- and **(Bb, Bd)** DMSO- soluble cAMP agonists in the same protein extracts represented in (A), as determined via western blotting, for which data is presented as mean ± SEM of the sum of values for low and high bands detected together (n = 5). For both (A) and (B), statistically significant differences are denoted by * ($p < 0.05$) and 'P=0.06' for borderline significance ($p = 0.0625$ for 1 mM 8-bromo-cAMP *versus* H_2O in (Aa); $p = 0.0606$ for 1 mM 8-bromo-cAMP *versus* H_2O in (Ba)). Representative western blot images for Ser16-phosphorylated ('P-HSP20') and total ('T-HSP20') HSP20 protein levels are shown below their corresponding graphs, alongside their matched glyceraldehyde 3-phosphate dehydrogenase (GAPDH) loading controls, with the same labels representing each treatment as used for representative PKA assay gel images shown in (A); positions of 'high' (thick arrow) and 'low' (thin arrow) HSP20 bands are also indicated.



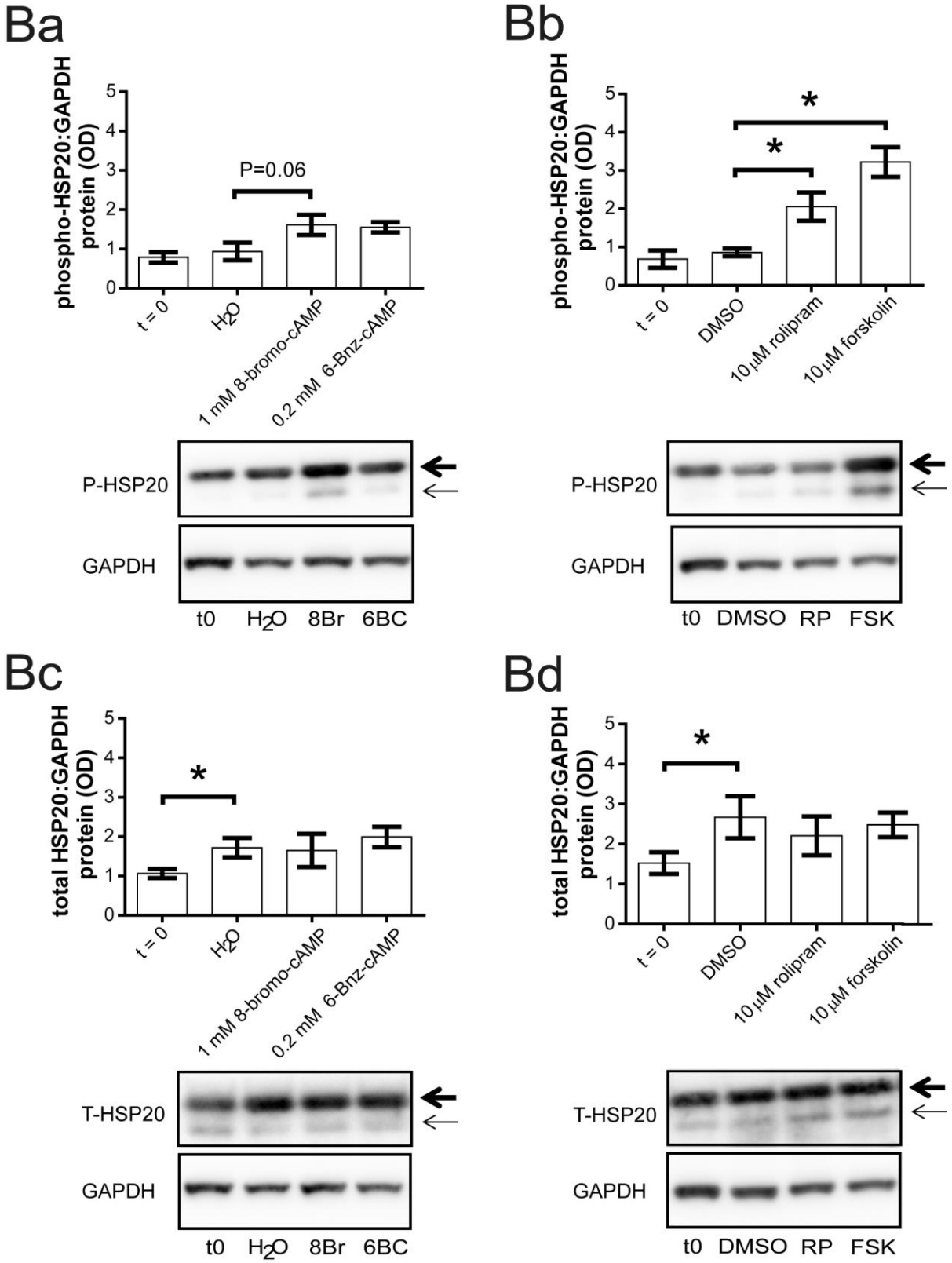


Figure 4. Treatment of pregnant human myometrial tissue for 24 hours with 8-bromo-cAMP simultaneously enhanced spontaneous contractions and PKA activity. Ability of myometrial tissues to contract both **(Aa)** spontaneously and subsequently **(Ab)** in response to oxytocin (1 nM Syntocinon®) measured after 24 hours treatment with cAMP agonists or vehicle controls, presented as absolute mean integral tension (MIT). Spontaneous contractions measured from 50 minutes immediately before oxytocin addition. Oxytocin-augmented contractions measured from last 30 minutes of 1 hour incubation with the hormone. **(Ac)** Representative traces of contractile output measured from tissues pre-incubated for 24 hours with 8-bromo-cAMP or vehicle control, showing contractions before and during, as indicated by the horizontal line, 1 nM oxytocin stimulation. **(B)** Protein kinase A (PKA) activity contents in protein extracts of the same tissues represented in (A) calculated as % phosphorylation of total assay substrate; Statistically significant differences denoted by * ($p < 0.05$) for both (A) and (B). Data presented as mean ± SEM ($n = 6$) for all graphs.

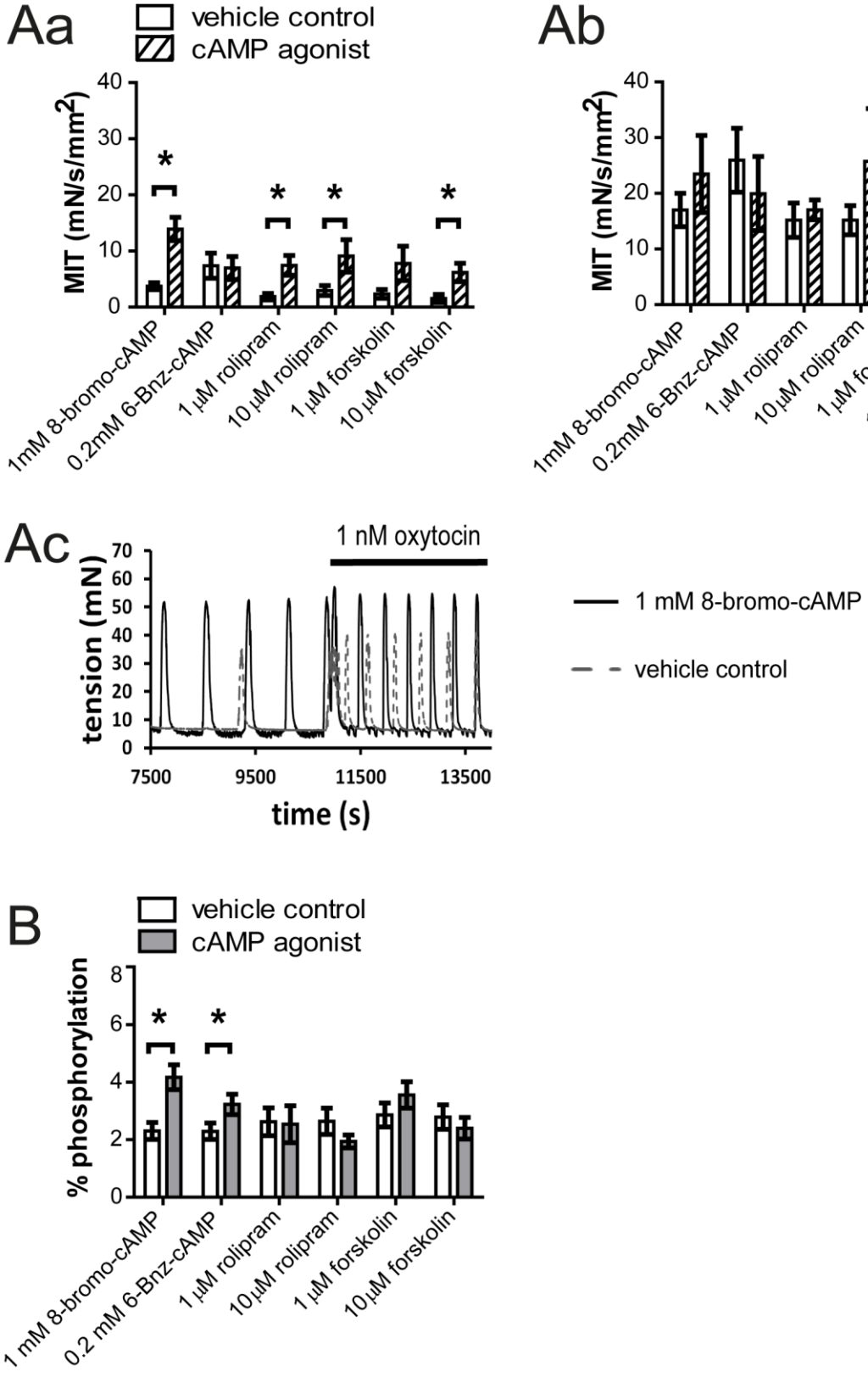


Figure 5. Enhanced spontaneous contractions after 24 hours treatment of pregnant human myometrial tissue with 8-bromo-cAMP was associated with enhanced calponin protein abundance but minor changes for both HSP20 and cofilin phosphorylation levels. Total protein extracts of tissues treated for 1 hour with DMSO (i.e. PF 04418948 vehicle control) during spontaneous contractions, as represented in Figure 7C, were measured for **(A)** total protein kinase A (PKA) activity content, as well as protein abundance of **(B)** calponin, **(C)** heat shock protein 20 (HSP20) Ser16-phosphorylation, **(D)** total HSP20, **(E)** cofilin Ser3-phosphorylation and **(F)** total cofilin. For phospho- and total HSP20 protein graphs, data is shown as the sum of values for low and high HSP20 bands detected together at western blots. All values presented as mean ± SEM for PKA activity (n = 6) and western blotting (n = 5) data; statistically significant differences denoted by * ($p < 0.05$). A typical agarose gel image of phosphorylated substrate from a PKA activity assay represented by (A) is shown above its associated graph. Representative western blot images for calponin, Ser16-phosphorylated HSP20 ('P-HSP20'), total HSP20 ('T-HSP20'), Ser3-phosphorylated cofilin ('P-cofilin') and total cofilin ('T-cofilin') are shown above their associated graphs, alongside matched images for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β-tubulin loading controls; positions of 'high' (thick arrow) and 'low' (thin arrow) HSP20 bands are also indicated. For all representative images of agarose gels and western blots, paired samples of tissues pre-incubated for 24 hours with H_2O ('C') or 1 mM 8-bromo-cAMP ('T') prior to isometric tension measurements for spontaneous contractile output are shown.

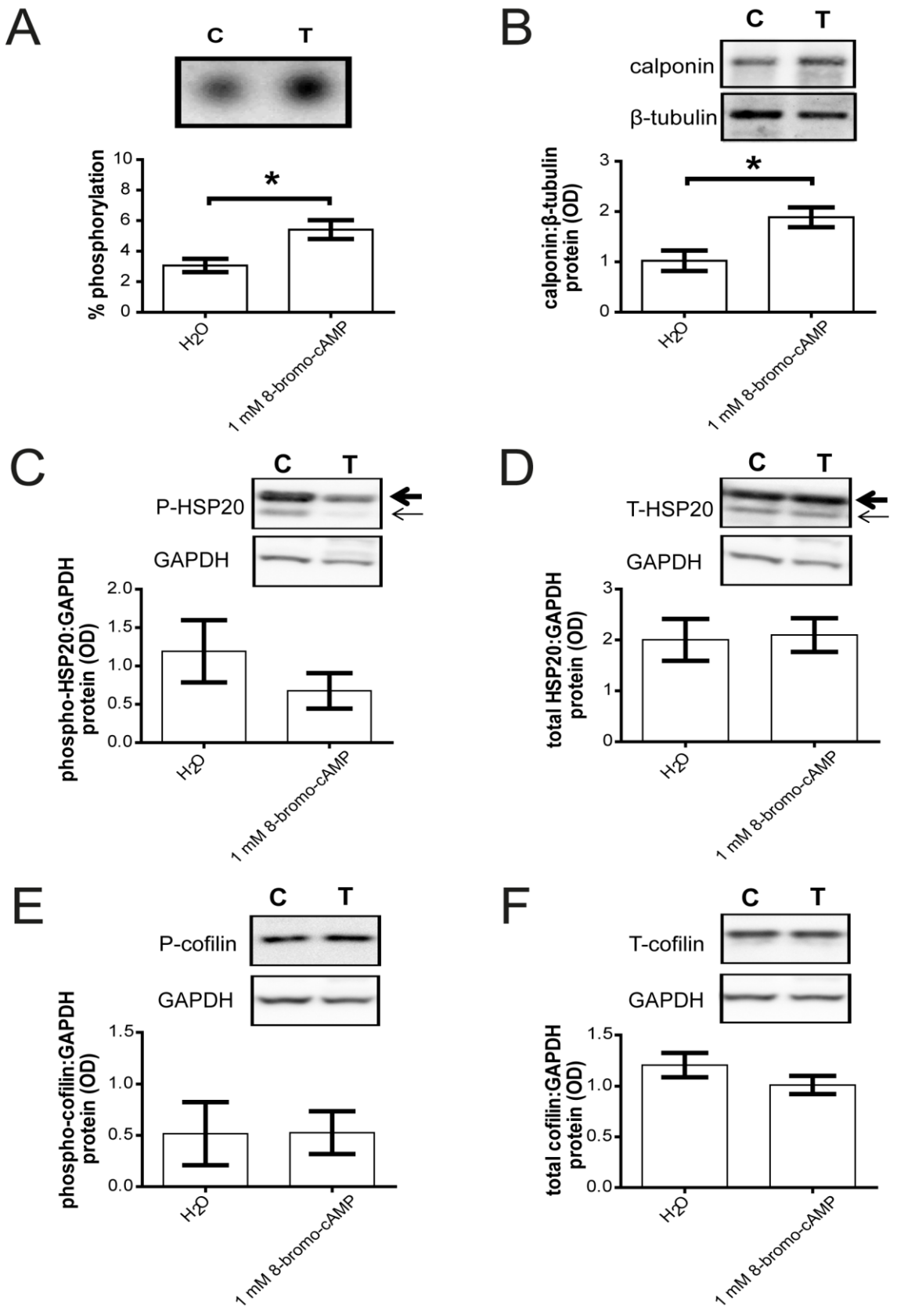


Figure 6. Treatment of pregnant human myometrial tissues for 24 hours with 8-bromo-cAMP and rolipram, with subsequent contractility measurements, reduced levels of HSP20 Ser16-phosphorylated and total cofilin proteins. (Aa) Ser16-phosphorylated and (Ab) total heat shock protein 20 (HSP20) protein levels were assessed by western blotting; protein extracts were prepared from tissues pre-incubated for 24 hours with cAMP agonists or their vehicle controls, and subsequently used for isometric tension measurements for both spontaneous and oxytocin-induced contractions (i.e. same samples represented in Figure 4). Each graph shows the sum of values for low and high HSP20 bands detected together. **(Ac)** Representative images of western blots for Ser16-phosphorylated ('Phospho-HSP20') and total HSP20 protein, alongside their sample-matched glyceraldehyde 3-phosphate dehydrogenase (GAPDH) loading controls; positions of 'high' (thick arrow) and 'low' (thin arrow) HSP20 bands for pair-matched vehicle control ('C') and cAMP agonist ('T') pre-incubated samples are indicated next to each immunoblot image. **(B)** Total cofilin protein levels were also measured via western blotting for all 6 treatment conditions represented in Figure 4; corresponding data shown alongside representative western blot images for total cofilin and sample-matched GAPDH. Data presented as mean ± SEM for all graphs, where n = 3-4 for (Aa) and (Ab), n = 5 for (B); statistically significant differences between cAMP agonist and vehicle treatment denoted by * ($p < 0.05$) and, in the case of (Aa) and (Ab), p values also given where n = 4 but did not pass the $p < 0.05$ threshold.

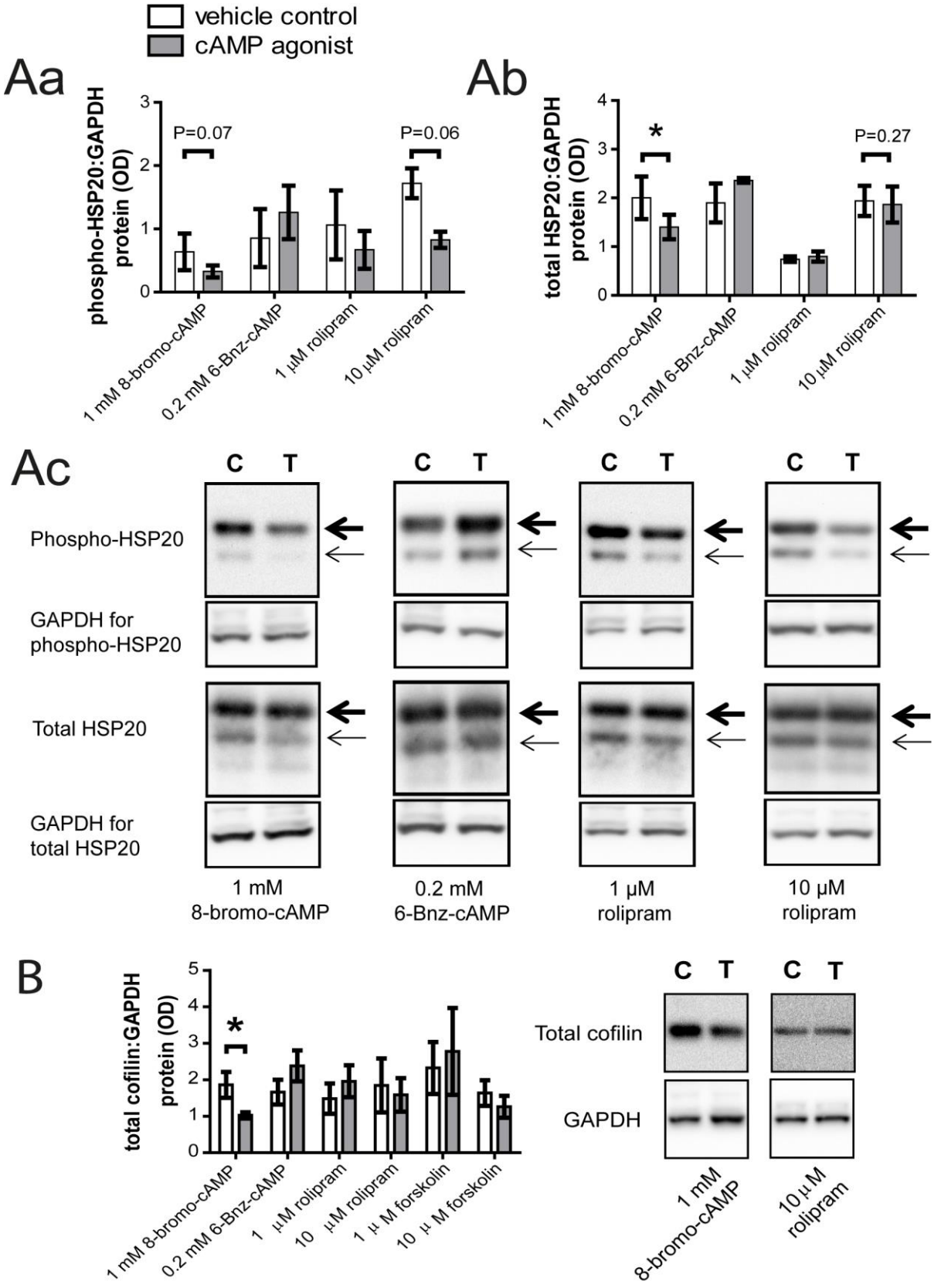


Figure 7. PTGER2 mRNA levels are increased after 24 hours treatment with 8-bromo-cAMP and rolipram, but did not correlate with a change to EP2 function in pregnant human myometrial tissue. Abundance of **(A)** cyclooxygenase-2 **(**COX-2) protein and **(B)** PGE_2 receptor subtype 2 (PTGER2, known as EP2 receptor at the protein level) mRNA from homogenate extracts of the same tissues represented in Figure 4 as determined using western blotting and qPCR, respectively. Data presented as mean ± SEM; statistically significant differences for COX-2 protein (n = 5) and PTGER2 mRNA (n = 6) are denoted by * ($p < 0.05$). For qPCR data at (B), the threshold cycle ('C_T') range for PTGER2 is also stated. **(C)** A separate set of tissues pre-incubated with 8-bromo-cAMP or its vehicle ('H_2O') for 24 hours prior to isometric tension measurements, from which spontaneous mean integral tension (MIT) was calculated from 30 minutes immediately before addition of, and final 30 minutes of incubation with, either EP2 receptor-specific antagonist, 10 μM PF 04418948 ('10 μM PF') or its vehicle ('DMSO'). Statistically significant differences were identified for the following comparisons: **(Ca)** MIT between 8-bromo-cAMP and H_2O vehicle pre-incubated strips before addition of PF 04418948 or DMSO vehicle, denoted by 'a' ($p < 0.05$), and MIT before and after treatment with PF 04418948, denoted by 'b' ($p < 0.05$); **(Cb)** change in MIT ('ΔMIT') after PF 04418948 treatment, denoted by * ($p < 0.05$). All MIT data presented as mean ± SEM (n = 8). **(Cc)** Representative traces of contractile output measured from tissues before and during, as indicated by the horizontal line, 10 μM PF 04418948 ("PF044") treatment.

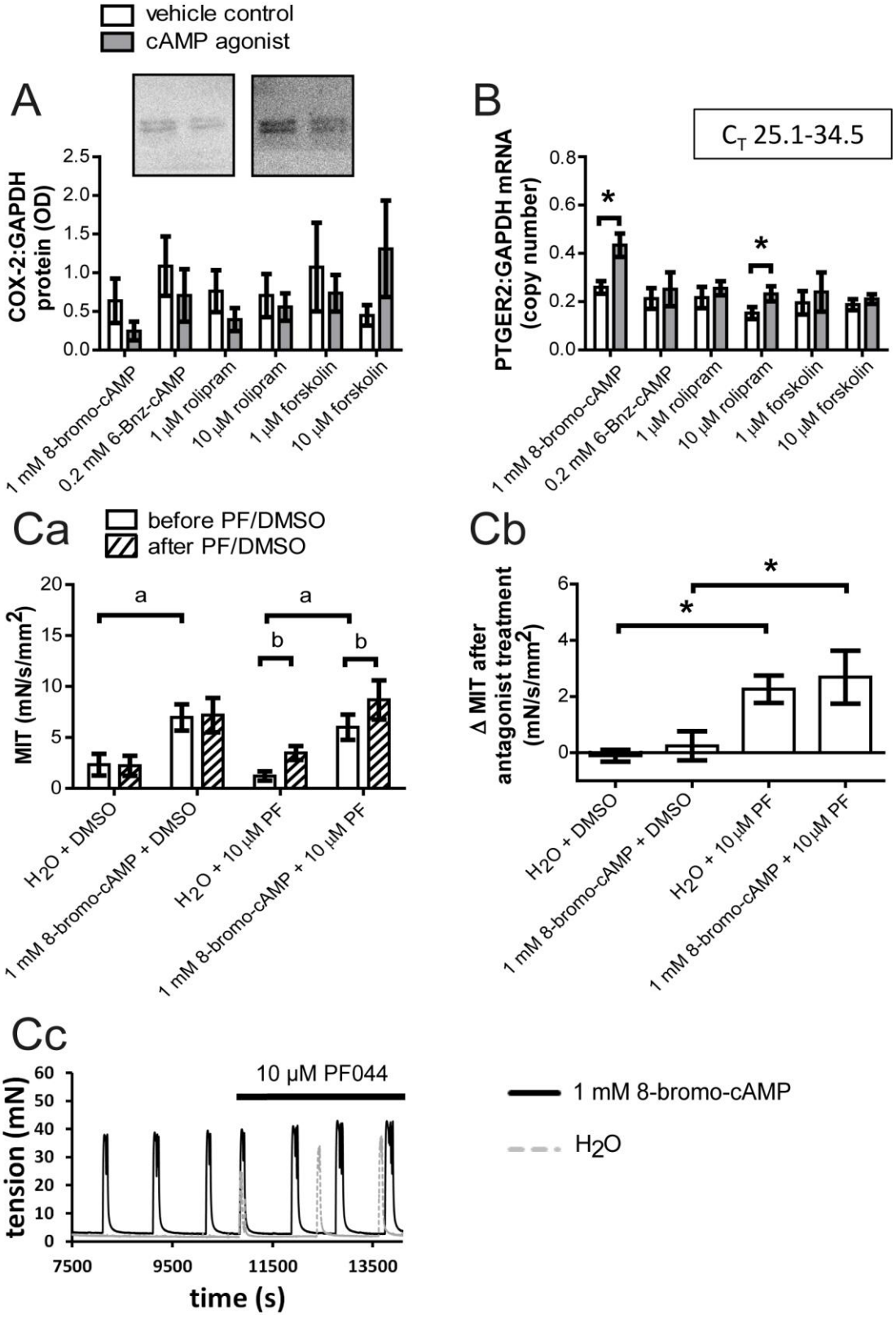


Figure 8. Dose-dependent spontaneous contractile responses to L-type calcium channel agonist Bay K8644 by pregnant human myometrial tissues were not significantly altered after 24 hours treatment with 8-bromo-cAMP. Spontaneous contractility of tissues pre-incubated for 24 hours with either 8-bromo-cAMP or its H_2O vehicle prior to and after non-cumulative dosing with Bay K8644. **(A)** Mean integral tension (MIT) calculated from 15 minutes prior to starting dosing with either Bay K8644 or its DMSO vehicle (indicated in brackets); statistically significant differences are denoted by * ($p < 0.05$). **(Ba)** Difference in MIT (ΔMIT) between the last 15 minutes of exposure to each corresponding dose of Bay K8644 or DMSO vehicle and last 15 minutes of spontaneous MIT prior to first dose of Bay K8644 (10^{-9} M) or DMSO vehicle (i.e. MIT values presented in (A)). **(Bb)** ΔMIT corrected for per contraction measured during treatment with associated Bay K8644 dose (instead of per unit time), and **(Bc)** contraction frequency during time period analysed. Statistically significant differences for all figures in (B) were not found in all cases ($p > 0.05$). All data presented as mean ± SEM ($n = 8$) for all graphs. **(Bd)** Representative traces of contractile output measured from tissues before and during Bay K8644 treatment, with arrows indicating time points at which a dose of the LTCC agonist was added.

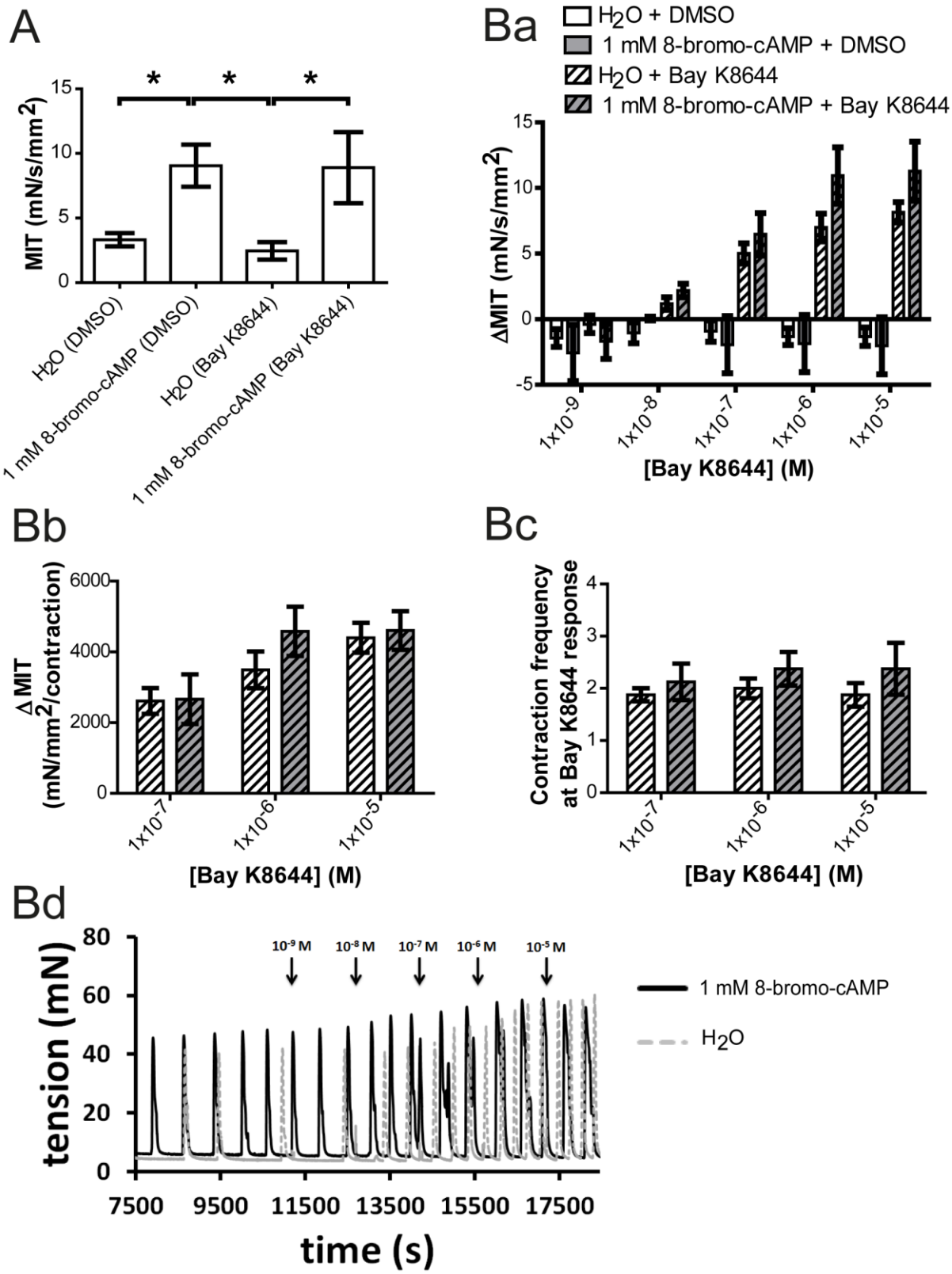


Figure 9. Directions of enhanced cAMP activity flow upon cAMP agonist treatment in pregnant human myometrial tissue. Schematic diagram of cAMP signalling targets and their activation status within myometrial cells of intact tissue during stimulation with cAMP agonists (labelled on the left of each associated scenario). Basal (i.e. endogenous cAMP at resting state) and enhanced (i.e. endogenous and analogue-based cAMP action during stimulated state) activities are indicated by the empty and filled shapes, respectively. Adenylate cyclase ('AC'), protein kinase A ('PKA') and phosphodiesterase 4 ('PDE4') are all established targets of signalling activity upon cAMP agonist treatment. Heat shock protein 20 ('HSP20') is a contractile apparatus-associated substrate (at its Ser16 residue) for both PKA and protein kinase G ('PKG'), though the activity of the latter is expected to be the lowest of the two kinases during exposure to all cAMP agonists tested. 'Exchange protein directly activated by cAMP' ('EPAC') has been included as a representative of alternative cAMP-binding proteins yet to have their actions in the myometrium fully elucidated.

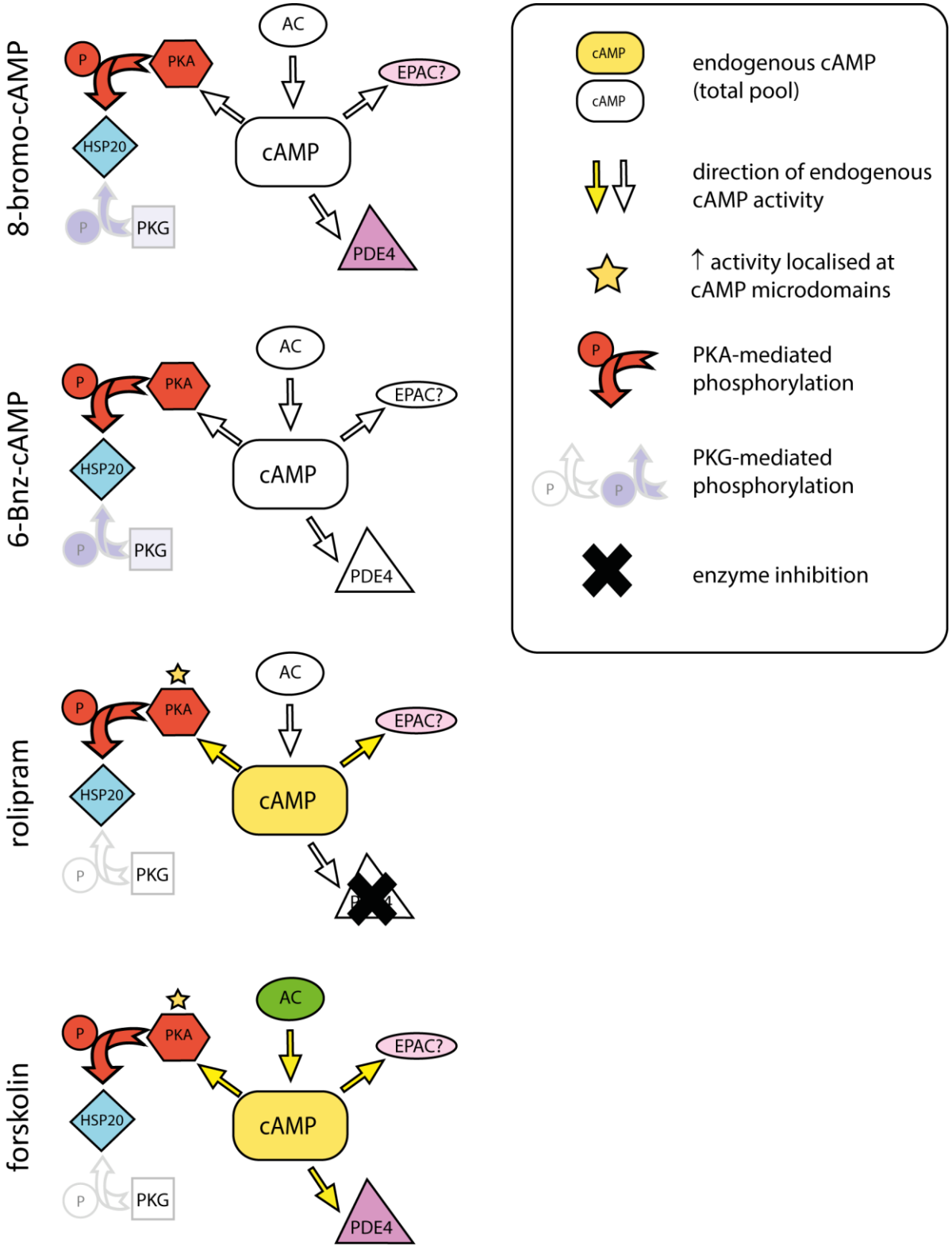


Figure 10. Proposed relationships between spatial distribution of enhanced cAMP signalling and its time-sensitive effects on spontaneous contractility in pregnant human myometrial tissue. Schematic diagram of cAMP signalling targets within a typical tissue-based myometrial cell (with the nucleus ('Nu') and endoplasmic reticulum ('ER') shown) and proposed distribution of enhanced cAMP signalling activity during 'acute' (1 h) and 'prolonged' (24 h) exposure to cAMP agonists (labelled on the left of each associated pair of scenarios). Their effects on spontaneous contractility are stated below each scenario, whereby the sizes of the arrows indicate relative intensity of change to contractile force. Basal (i.e. endogenous cAMP at resting state) and enhanced (i.e. endogenous and analogue-based cAMP action during stimulated state) activities are indicated by empty and filled shapes, respectively; shading within the cytosolic area indicate proposed intracellular locations of elevated cAMP-driven activity highly capable of acting on multiple targets. Proteins depicted are as follows: protein kinase A ('PKA'), adenylate cyclase ('AC'), heat shock protein 20 ('HSP20'), phosphodiesterase 4 ('PDE4'), A-kinase anchoring protein ('AKAP'), G protein-coupled receptor ('GPCR') and G protein α_s ($G\alpha_s$; $\beta\gamma$ subunits have not been included to simplify the diagram). The positions of PKA (specifically regulatory subunits), HSP20 and AKAP are inferred from previous myometrium-based studies, while that of PDE4 is speculated.

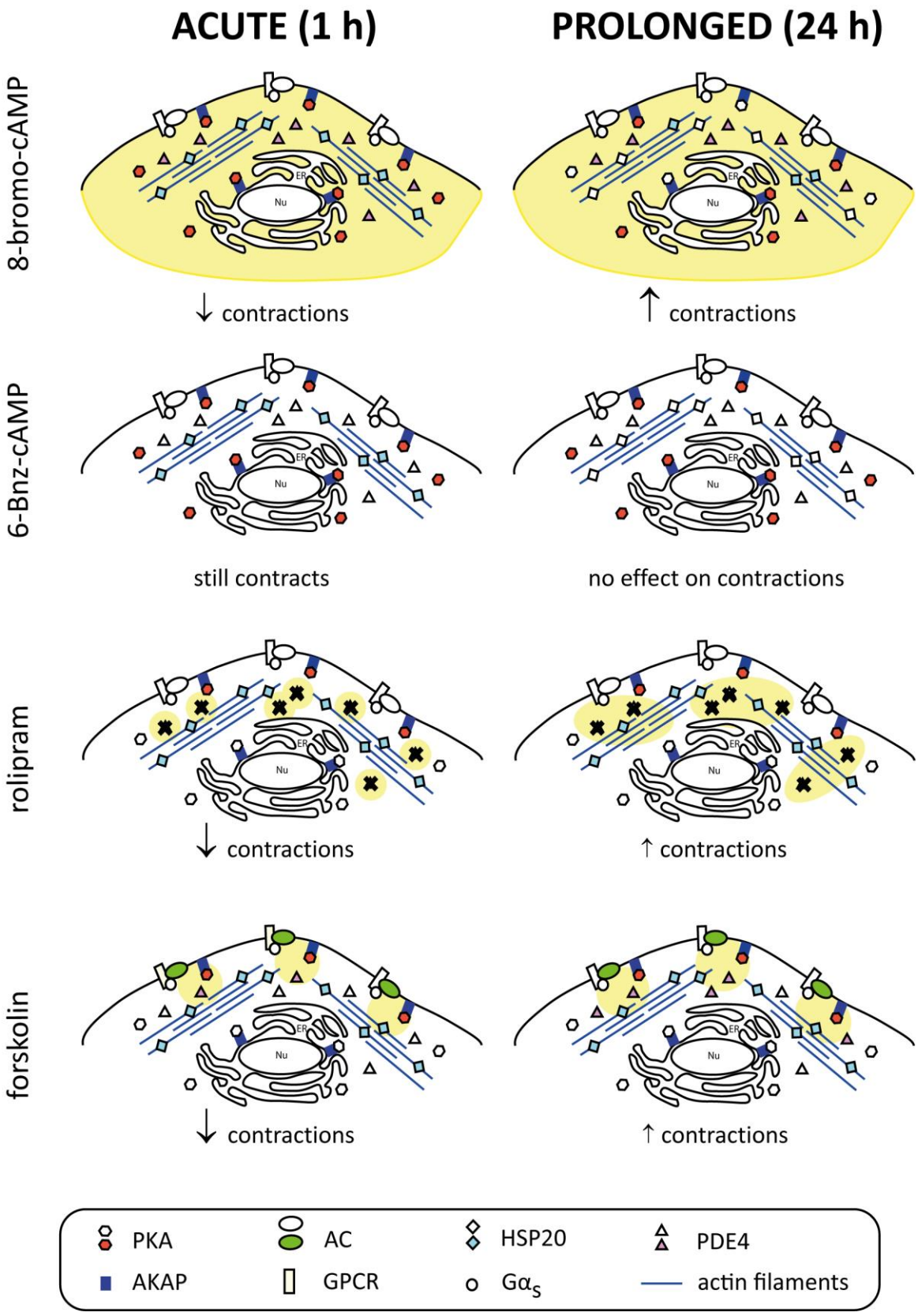


Table 1: Patient Demographics for Myometrial Tissue Samples

	1 h acute treatment (data presented: n = 49; qPCR templates for standards: n = 2)	**24 h pre-incubation (data presented: n = 36)**
Maternal age (years; median with range)	34 (21-46)	36 (26-46)
Gestational age (weeks; median with range)	39 (37.7-41.6)	39 (38.0-41.1)
Gravida (median with range)	2 (1-7)	2 (1-8)
Parity (median with range)	1 (0-3; live births) + 0 (0-4; non-viable & abortus)	1 (0-3; live births) + 0 (0-6; non-viable & abortus)
Booking BMI (median with range)	23 (17-42)	23 (18-33)
Ethnicity	• 34 White European • 6 White other • 2 Black African & Caribbean • 2 Asian • 4 Other • 3 Not disclosed	• 18 White European • 0 White other • 5 Black African & Caribbean • 5 Asian • 4 Other • 4 Not disclosed
Reason for Caesarean	• 25 Previous Caesareans • 15 Breech • 8 Other maternal clinical indication (GDM and PET excluded from study) • 2 Maternal request • 1 Tocophobia	• 22 Previous Caesareans • 4 Breech • 7 Other maternal clinical indication (GDM and PET excluded from study) • 3 Maternal request • 0 Tocophobia

Primary antibody target	**Supplier (catalog no.)**	**Dilution**
Phospho (Ser16)- heatshock protein 20 (HSP20)	Abcam (ab59522)	1:10,000
Total heat shock protein 20 (HSP20)	Abcam (ab184161)	1:80,000
Connexin-43(Cx43)	New England BioLabs (#3512)	1:1,000
Oxytocin receptor (OTR)	Santa Cruz (sc-8102)	1:1,000
Cyclooxygenase-2 (COX-2)	Santa Cruz (sc-1745)	1:1,000
Calponin	Abcam (ab46794)	1:20,000
Sarco/endo-plasmic reticulum calcium ATPase 2 (SERCA2)	Abcam (ab2817)	1:1,000
Smooth muscle myosin heavy chain (SM-MHC)	Millipore (MAB3568)	1:10,000
Phosphodiesterase 4B (PDE4B)	Abcam (ab112014)	1:500
Protein kinase A regulatory subunit 2α (PKA RIIα)	Abcam (ab38949)	1:500
G protein α_s ($G\alpha_s$)	Abcam (ab83735)	1:4,000
Phospho (Ser3)-cofilin	New England BioLabs (#3311)	1:500
Total cofilin	New England BioLabs (#3312)	1:500
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Millipore (MAB374)	1:40,000
β-tubulin	Abcam (ab21057)	1:1,000

Table 2: Primary Antibodies for Western Blotting

Primary antibody target	**Supplier (catalog no.)**	**Dilution**
Phospho (Ser16)- heat shock protein 20 (HSP20)	Abcam (ab58522)	1:10,000
Total heat shock protein 20 (HSP20)	Abcam (ab184161)	1:80,000
Connexin-43(Cx43)	New England BioLabs (#3512)	1:1,000
Oxytocin receptor (OTR)	Santa Cruz (sc-8102)	1:1,000
Cyclooxygenase-2 (COX-2)	Santa Cruz (sc-1745)	1:1,000
Calponin	Abcam (ab46794)	1:20,000
Sarco/endo-plasmic reticulum calcium ATPase 2 (SERCA2)	Abcam (ab2817)	1:1,000
Smooth muscle myosin heavy chain (SM-MHC)	Millipore (MAB3568)	1:10,000
Phosphodiesterase 4B (PDE4B)	Abcam (ab112014)	1:500
Protein kinase A regulatory subunit 2α (PKA RIIα)	Abcam (ab38949)	1:500
G protein α_s ($G\alpha_s$)	Abcam (ab83735)	1:4,000
Phospho (Ser3)-cofilin	New England BioLabs (#3311)	1:500
Total cofilin	New England BioLabs (#3312)	1:500
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Millipore (MAB374)	1:40,000
β-tubulin	Abcam (ab21057)	1:1,000

Table 3: Primer sequences for qPCR

Name of mRNA template	Accession number	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')	Amplicon size (bp)	qPCR C_T range
PGE_2 receptor subtype 1 (PTGER1)	NM_000955.2	AACCAGATCCTGGACCCTTG	GGCCTCTGGTTGTGCTTAGAA	198	n/a
PGE_2 receptor subtype 2 (PTGER2)	NM_000956.3	GCTCCTTGCCTTTCACGATTT	AGGATGGCAAAGACCCAAGG	127	25.1-34.5
PGE_2 receptor subtype 3 (PTGER3)	NM_001126044.1	ATGACTGTTTTCGGGCTCTC	GGCCACTGGACGGTGTACT	203	29.8-32.2
PGE_2 receptor subtype 4 (PTGER4)	NM_000958.2	ACAGCCACTACGTGGACAAG	ACAAGCACGTTGCAGAGGAC	238	n/a
PGE synthase 1 (PTGES)	NM_004878.4	GAAGAAGGCCTTTGCCAACCC	GTGCATCCAGGCGACAAAAG	187	32.1-35.9
PGE synthase 2 (PTGES2)	NM_025072.6	GTGGAATCCCATCCCTGGGT	ATGACAGGCGCCACAAACC	100	31.9-33.4
PGE synthase 3 (PTGES3)	NM_006601.6	CGACCGGAGAGAAAAAGCGG	GCAGGCTGCATTGTGAACGG	122	24.3-27.2
$PGF_{2\alpha}$ receptor (PTGFR) 1	NM_000959.3	TGGAGCCCATTTCTGGTTACA	TGCACTCCACAGCATTGACT	194	24.7-39.4
$PGF_{2\alpha}$ receptor (PTGFR) 2	NM_001039585.1	GTAATCCAGCTCCTGGCGAT	CAATGTTGGCCATTGTAACCTATG	147	n/a
$PGF_{2\alpha}$ synthase (PGFS)	NM_003739.5	ATTTGGCACCTATGCACCTC	CACACTGCCATCTGCAATCT	163	30.5-33.4
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	NM_002046.5	TGATGACATCAAGAAGGTGGTGAAG	TCCTTGGAGGCCATGTAGGCCAT	240	19.3-30.7